Genetic Characterisation of Canine and Equine Papillomaviruses and Papillomatoses

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Summary

Papillomaviruses can infect epithelia of humans and animals, resulting in most cases in asymptomatic infections. However many benign and several malignant disorders were shown to result from papillomavirus infections. Many different papillomaviruses have been described, most of them infecting humans. Animals have their own papillomaviruses but relatively few animal papillomaviruses are characterized or known at all. Even in common domestic animals, like the dog and the horse only one papillomavirus each had been studied in some detail in the past. This study was consequently initiated to shed more light onto the papillomavirus situation in these two species and to establish viable tools for this purpose. We were able to identify novel canine papillomaviruses (CPVs) associated with distinct clinical conditions. The DNA genomes from some of these viruses were cloned and sequenced. We found, that histologically distinct types of inverted papillomas seem to be associated with the presence of DNA belonging to different CPVs. Sequence analyses of newly cloned CPV genomes revealed that these genomes belonged to the papillomavirus genera Lambda, Tau and Chi. This phylogenetic characterization was supported by analysis of other features, such as genome organization and genome size. Some clinical-pathological characteristics were also reflected in this categorization, above all the finding that all CPVs inducing pigmented plaques belonged to the Chi papillomavirus genus. Based on a careful evaluation of PCR assays it was possible to show that viral DNA could be detected in the clinically healthy tissue from oral mucosa and interdigital skin of dogs. As a first step towards developing a tool for future research on the mechanisms of virus cell interaction, we have in addition generated keratinocyte cell lines harboring and transcribing the DNA of CPV1, CPV3 or CPV5 respectively. We also conducted a related project on horse papillomaviruses. In the horse papillomatoses are a well known problem and have already been described in ancient times. Curiously, the best known papillomavirus affecting horses so far is a bovine papillomavirus, BPV1, involved in the equine sarcoid. In this project, we identified equine papillomaviruses (EcPVs) in two other occasionally observed disorders, namely penile papillomas and aural plaques. We established PCR and in situ hybridisations and showed that samples of penile papillomas from the histological archive were positive for either of two described variants of EcPV2. Viral DNA was located in the nuclei of koilocytes, cells typical for active viral infection in the skin. This test might help in the molecular classification of equine papillomatoses.

Zusammenfassung

Papillomaviren können Epithelien wie vornehmlich Haut und Schleimhäute von Mensch und Tier infizieren, was meist zu asymptomatischen Infektionen führt. Papillomaviren stehen nichts desto trotz auch mit vielen gutartigen sowie einigen bösartigen Tumoren in Verbindung. Obgleich die meisten derzeit bekannten Papillomaviren den Menschen betreffen, haben auch Tiere ihre eigenen Papillomaviren. Tierpapillomaviren kennt man allerdings bisher nur bei einer Hand voll Spezies und auch jeweils nur wenige Vertreter. Über die Biologie der Papillomaviren bei Tieren ist in den meisten Fällen kaum etwas bekannt. Selbst bei weit verbreiteten Haustierspezies wie dem Hund und dem Pferd wurde jeweils gerade ein Papillomavirus etwas genauer studiert. Folglich zielte unsere Studie darauf ab, die Papillomavirus Situation in diesen beiden Spezies etwas genauer zu beleuchten und in diesem Zusammenhang brauchbare Techniken für die weitere Erforschung zu etablieren. Wir konnten neue mit typischen Veränderungen assoziierte Hundepapillomaviren (CPVs) identifizieren und einige von diesen wurden kloniert und sequenziert. Wir konnten zeigen, dass bestimmte histologisch unterschiedliche Arten von invertierten Papillomen auch mit unterschiedlichen Arten von CPV DNA in Verbindung zu stehen scheinen. Die Analyse der Gensequenzen von neu entdeckten und klonierten CPVs liess eine Zuordnung zu den Papillomavirus Genera Lambda, Tau und Chi zu. Diese phylogenetisch basierte Zuordnung wurde durch andere genetische Charakteristika wie Genomorganisation oder Grösse unterstrichen. Auch einige typischerweise zu beobachtende klinisch-pathologische Charakteristika einzelner Krankheitsbilder wurden von dieser Zuordnung wiedergespiegelt, in erster Linie die Versammlung aller mit flachen pigmentierten Warzen (pigmentierten Plaques) assoziierten CPVs im Chi Genus. Basierend auf einer gründlichen PCR Evaluation konnte des Weiteren gezeigt werden, dass virale DNA auch auf klinisch gesunder Haut der Maulschleimhaut und in Zwischenzehenspalt des Hundes vorkommt. Um ein Model für zukünftige Studien für Virus-Zell Interaktionen zu haben, wurden Keratinozyten-Zelllinien etabliert, die die DNA der CPVs 1, 3 oder 5 tragen und diese auch übersetzen. Auch beim Pferd sind Papillomatosen bereits seit der Antike bekannt. Das am besten beschriebene Papillomavirus in diesem Zusammenhang ist jedoch eines des Rindes (BPV1), welches beim Equinen Sarcoid eine Rolle spielt. Wir konnten weitere Pferdepapillomaviren (EcPVs) in zwei weiteren Arten von gelegentlich auftretenden Veränderungen identifizieren, nämlich bei Penispapillomen und

flächig flachen Ohrwarzen (auralen Plaques). PCR und in situ Hybridisierung wurden etabliert, um mehr Aufschluss darüber zu erlangen, welche Rolle die Papillomaviren in solchen Penisläsionen spielen. Diese Methoden wurden dann angewendet um entsprechende Proben aus dem histologischen Archiv zu testen. Alle getesteten Proben enthielten DNA einer von zwei EcPV2 Varianten. Die DNA war in den Kernen der Koilozyten lokalisiert, die typisch für virale Infektionen sind.

Introduction

Viruses

Viral diseases can be traced back through the whole human history, but viruses themselves were only discovered in the late 19th century. The identification of the tobacco mosaic virus as the first plant and foot and mouth disease virus as the first animal virus mark the beginning of virus research (Mayer, 1886; Iwanowsky, 1892; Loeffler, 1897; Loeffler, 1898). However the viral nature of micro filterable sub microscopic pathogens was for several decades all that was known. The development of electron microscopy and molecular biological techniques during the 20th century contributed decisively to the understanding of virus structure and biology (Bernal, 1941; Black, 1963). The knowledge about viruses and viral diseases has consequently increased vastly to the present day. Although there are enormous differences among different kinds of viruses they share some common features: Each virus is build of at least the viral capsid (protein) and the genetic information (RNA or DNA) contained therein. Based on this genetic information and the mechanisms used to replicate it, viruses can be allocated to seven categories, in particular double stranded DNA viruses (I), single stranded DNA viruses (II), double stranded RNA viruses (III), positive strand single strand RNA viruses (IV), negative strand single strand RNA viruses (V), single strand RNA viruses with reverse transcriptase activity (VI) and double stranded DNA viruses with reverse transcriptase activity (VII) (Baltimore, 1971; Temin, 1972). In the overall virus taxonomy properties like morphology and sequence homology are used as additional criteria to define for example order and family. The criteria below that level may however differ significantly. While in some virus families serotype designations are used other viruses like papillomaviruses are primarily classified according to their nucleic acid sequence (Bernard, 2010).

Viruses differ from other pathogens like eukaryotic parasites, funguses and bacteria not only in terms of size. A main difference is that all viruses are obligate parasites of cells. They dependent entirely on the host cell to provide all resources for viral reproduction, and also to a variable degree on the cellular machinery. Viruses may therefore be described as being “at the edge of life”, as they fulfil most but not all current criteria of a life form (Rybicki, 1990). Due to their relative simplicity several theories about the origin of viruses have been formulated including the hypothesis, that viruses are the closest relatives to the earliest life forms and contrary views regarding them as degenerate parasites or genes that became independent. Consequently their role in evolution has been and is controversially discussed and there are

various arguments pro and contra an inclusion of viruses in the tree of life (Podolsky, 1996; Forterre, 2002; Moreira, 2009; Ludmir, 2009; Forterre, 2010).

Viruses and cancer

Although viruses may have often be perceived as pathogens directly inducing obvious diseases, that it is not the only role they play. While apparent viral induced maladies like foot and mouth disease or small pox caught the attention of virologist from the beginning on, other more complex or less obvious effects of viral infections moved into the focus more recently (Loeffler, 1898; Fenner, 1988; zur Hausen, 2002). Not long after the existence of viruses infecting plants and animals had been demonstrated, the hypothesis that viruses could be involved in cancer development was already formulated. It was based on the finding of transmittable tumours in chickens and confirmed later (Ellermann, 1908; Rous, 1911; Bryan, 1955). Today the etiology of a series of malignant neoplasias is known, in which viruses are essentially involved including feline leukemia, the hepatocellular carcinoma, Burkitt's lymphoma and cervix carcinomas. Although it is believed that viruses play a role in the development of at least 12% of all cancers in humans, malignant transformation is not a necessary part of virus life cycle but an accidental side effect (zur Hausen, 2001; Parkin, 2006; de Martel, 2009). Consequently the mechanisms involved in this process differ among the viruses and even virus types. Integration of viral DNA that can turn cellular proto-oncogenes into oncogenes as in case of retroviruses is a common feature, but not universal for tumour viruses. The interaction of oncogenic viral proteins with the cellular machinery is very common in case of DNA tumour viruses.

Although the infection is a mandatory element in virus induced oncogenesis, other factors are usually involved. One such factor could be an acquired immunodeficiency like HIV-AIDS that can promote the development of virus induced tumours like Kaposi's sarcoma, Burkitt's lymphoma or cervical cancer (International Collaboration on HIV and Cancer, 2000). However the immune reaction may as well contribute to tumourous developments as is discussed in case of hepatitis B (Sanyal, 2010). Another factor could be a genetic predisposition as demonstrated in the case of epidermodysplasia verruciformis (Jablonska, 1972).

Double stranded DNA viruses

Viruses carrying their genetic information in form of double stranded DNA are manifold and to date two orders, nineteen Families and five unassigned genera belong to this group (Fauquet, 2005). However, virus classification is still an ongoing process as the ongoing research is constantly unveiling new genotypes, serotypes and entirely novel viruses. Among the group of double stranded DNA viruses are members with large genomes like the members of the herpesvirales or the mimiviridae which have the largest yet discovered viral genome and encode many viral genes. Nevertheless, there are also double stranded DNA viruses with genomes well below ten thousand basepairs like papillomaviruses or polyomaviruses coding for only few genes. Because these two both have circular genomes and due to similarities in capsid structure they were initially grouped together in the family *Papovaviridae* (Belnap, 1996). As genome size and organisation were found to differ and no significant sequence homologies could be identified, this categorisation was abandoned and the taxonomic families *Polyomaviridae* and *Papillomaviridae* were established. However, some similarity exists in the encoded helicase motives and recently a virus with papillomavirus and polyomavirus features was discovered (de Villiers, 2004; Woolford, 2007).

Papillomaviruses

Papillomaviruses consist of a non-enveloped icosahaedral capsid and their doublestranded DNA genome contained therein. The capsid is comprised of the viral proteins L1 and L2 and has a diameter of 50-55 nm (Figure 1) (Crawford, 1963; Baker, 1991; Belnap, 1996). The genomes of papillomaviruses are circular and approximately eight thousand basepairs in length (Figure 2). Unlike in polyomaviruses all genetic information is coded on the same strand of the papillomavirus genome (Chen, 1982). Four open reading frames can easily be identified on this coding strand in all papillomaviruses, namely the early ones E1 and E2 as well as the late ones L1 and L2. Most papillomaviruses also contain the two additional early open reading frames E6 and E7 and in some papillomavirus genera an E5 open reading frame is also characteristic (de Villiers, 2004). There is also an E4 open reading frame located within E2, however the according start codon is located upstream of it. Other early open reading frames can be identified in a few papillomavirus types, where they often are replacing position and possibly functions of E6 and/or E7 (Tachezy, 2002; Terai, 2002).

A large non coding region downstream of the L1 open reading frame (usually between L1 and E6) harbours the origin of replication of the papillomaviruses; several have a second non coding region between E2 and L2 whose relevancy is unknown (Chen, 1982, Delius, 1994; Schulz, 2009a).

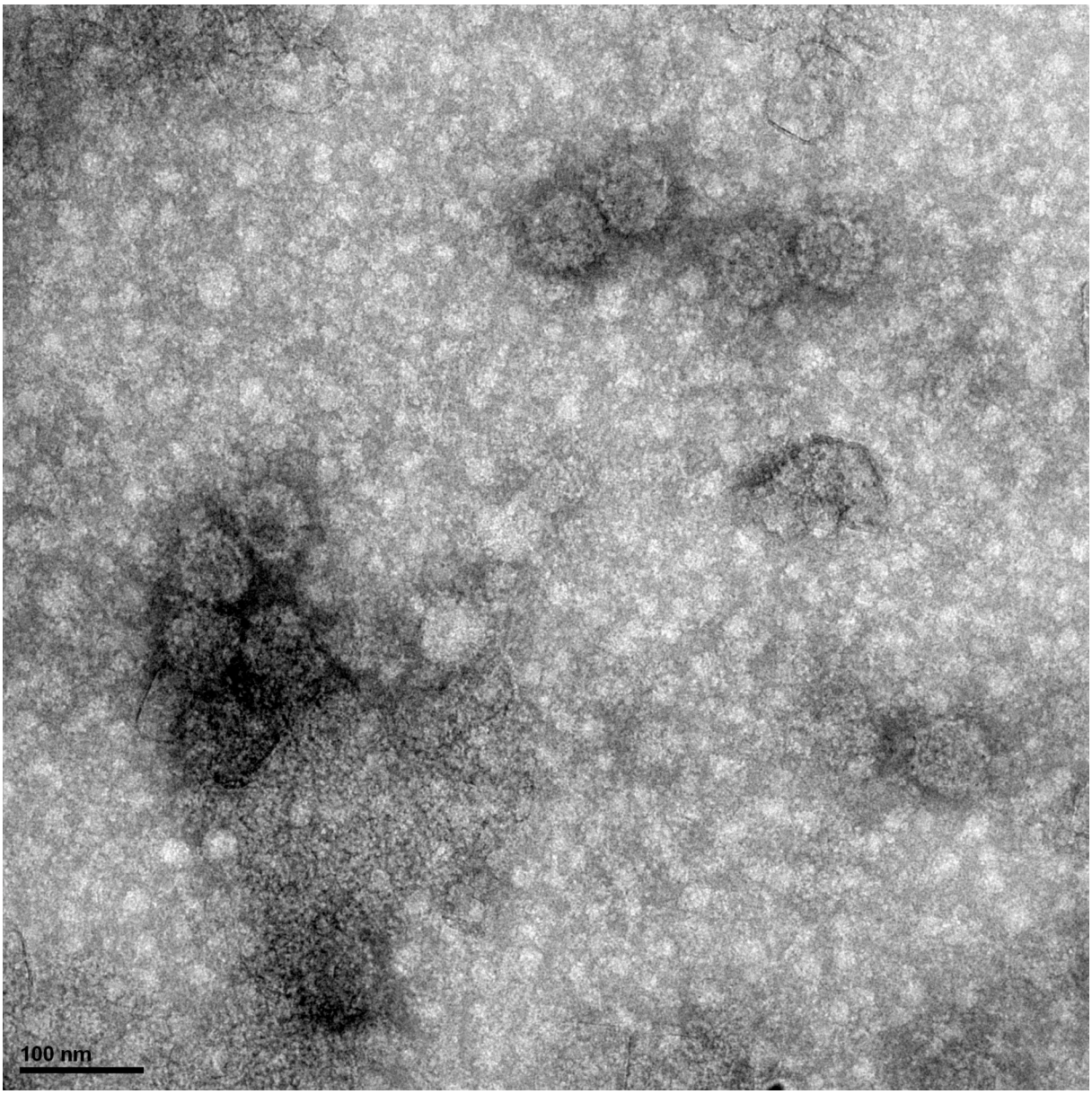

Image: E. M. Schraner, UZH

Figure 1

Canine papillomaviruses isolated from a pigmented lesion. Negative contrast electron microscopy.

Papillomavirus classification

The current classification of papillomaviruses within the *Papillomaviridae* family is based on nucleotide sequence identities in one of the papillomavirus genes, namely L1. The L1 open reading frame was chosen because it is the most conserved among the known papillomaviruses. This choice was also strongly supported by the observation, that phylogenetic relationships based on whole genomes or even parts of it differ only marginally from phylogenetic relationships based on L1 (de Villiers, 2004).

However, when criteria for genera (<60% identities), species (>60% identities), types (>70% identities), subtypes (>90% identities) and variants (>98% identities) were defined, complete sequences of only one hundred eighteen papillomaviruses were available, most of them being human papillomaviruses (de Villiers, 2004). Thus the usefulness of this single gene based classification in the context of papillomavirus evolution is controversially debated, as are the mechanisms of papillomavirus evolution. There is evidence that virus-host co-evolution is a driving force in papillomavirus diversification based mainly on the analysis of human papillomaviruses but also on the analysis of papillomaviruses of other closely related host species (Tachezy, 2002; Bernard, 2006; Rector, 2007). In contrast it could be demonstrated, that different genes have different evolutionary histories when papillomaviruses of more distant host species are taken into account (Gottschling, 2007; Stevens, 2008; Schulz, 2009a, b). Thus papillomavirus evolution is probably a complex matter involving different mechanisms.

At the moment the family of papillomaviridae contains 29 defined papillomavirus genera (Bernard, 2010), but the number of distinct papillomavirus types is growing constantly with already more than 200 being sequenced in their entity. The currently rapid accumulation of novel viral sequences constitutes a challenge for papillomavirus virus nomenclature. Thus just recently new names for additional genera were proposed and the nomenclature of several papillomavirus types was suggested to be changed in order to avoid misunderstandings and for more stringency (Bernard, 2010).

Papillomavirus genes

The inability to culture papillomaviruses has been one of the main obstacles in papillomavirus research and although various model systems have been established, there is still no suitable way for culturing papillomaviruses (Meyers, 1994; Conway, 2009; Liebertz, 2010). However, molecular cloning made it possible to analyse the genomes in detail and to establish various model systems for papillomavirus infections. It became possible to produce pseudovirions

consisting of one (L1) or both structural proteins as well as to study the interactions of viral and cellular proteins, thus linking papillomavirus gene products to pathogenesis (Kirnbauer, 1993; Hagensee, 1994). The nomenclature of papillomavirus genes is mainly based on the localisation of open reading frames and on sequence homology (Figure 2). While there are only few open reading frames that can easily be identified in papillomavirus genomes, excessive splicing has been shown to give rise to a variety of gene products fulfilling different functions in the viral lifecycle (Baker, 1987; Hummel, 1992; Stubenrauch, 1999). However, even with only few papillomaviruses studied in particular detail it has become clear, that not all these nominally similar genes give rise to proteins with identical functions. Especially as the papillomavirus gene products seem to interact with a great number of cellular proteins. It consequently appears that papillomaviruses developed different mechanisms throughout evolution, which may explain some of the differences in papillomavirus mediated diseases (Roberts, 2002; Howley, 2007).

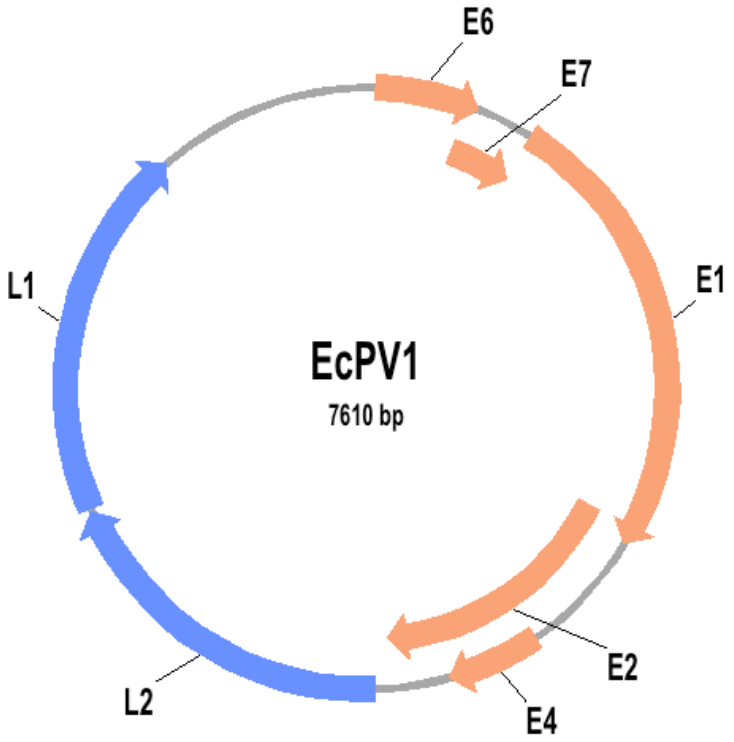

Figure 2

Image: Vector NTI Software

Schematic display of a papillomavirus genome; Equine papillomavirus type 1

Early genes

The gene products of E1 and E2 fulfil certain well defined functions in the papillomavirus life cycle and are therefore believed to be rather conserved in terms of sequence and function among the different papillomavirus types. E1 gene products are primarily involved in

replication, as the main product is a helicase that also has the ability to recruit replication initiation factors to the papillomavirus genome (Conger, 1999; Loo, 2004). E1 usually forms a complex with E2 that has accessory functions in the replication initiation (Masterson, 1998). Besides enhancer functions for the replication E2 products play a primary role in transcriptional control, being able to function as a transactivator as well as a repressor of the viral genes. Another role of E2 is the distribution of the papillomavirus genomes during cell division. In case of papillomavirus integration the E2 open reading frame is often destroyed or entirely lost, thus leading consequently to a loss of its functions, resulting for example in uncontrolled E6 and E7 expression (Zur Hausen, 2002).

The roles of the other early gene products are not as well conserved among the papillomaviruses and so are their nucleotide sequences. E6 and E7 open reading frames can be identified in most, E5 in certain clades of papillomaviruses as in those from the genus alpha (de Villiers, 2004). Each of these gene products has been found to have many different cellular targets, and research in this field is still ongoing (Zur Hausen, 2001; Regan, 2008). Due to their oncogenic potential the roles of E5, E6 and E7 have been studied in great detail in case of the human papillomavirus 16 (HPV16), consequently these mechanisms are often referred to. The most striking finding in HPV16 E5 is that it interacts with the epidermal growth factor receptor, thereby inducing accelerated cell growth (Crusius, 2000). With respect to HPV16 E6 it is well established, that its products are able to interact with the ubiquitin ligase E6AP which leads to the ubiquitination and degradation of the tumour suppressor p53. E6 products also interact with the proapoptotic protein Bak leading to its degradation (Jackson, 2000; Horner, 2004). The best studied mechanism regarding HPV16 E7 is its interaction with regulatory protein pRB, mediating its proteolytic degradation and in the consequence leading to E2F mediated transcriptional activation of cell cycle genes (Cheng, 1995).

Late genes

Two genes are defined as late genes in the papillomavirus genome, as they are expressed in late stages of a papillomavirus infection (Baker, 1987). However, as this is not experimentally determined for all papillomaviruses, these late genes are identified according to their position and due to similarities with late genes of other papillomaviruses. The two L genes give rise to the two proteins L1 and L2, both having structural functions. The L1 protein is the main component of the viral capsid. It forms pentamers that assemble to the 72 pentamer icosahedral structure of the papillomavirus capsid. This assembly takes place even in the

absence of the second structural protein L2, and this spontaneous assembly of viral capsids has been used for several applications including diagnostic and prophylactic ones (Segre, 1955; Kirnbauer, 1993; Hagensee, 1994; Kirnbauer, 1994; Harro, 2001). As L1 is much more abundant in the viral particle, it is also known a major capsid protein, while L2 is counted as minor capsid protein. However L2 is also a mandatory part of the infectious viral particle, as it plays a major role in uncoating and intracellular transport (Kämper, 2006). The protein derived from the E4 open reading frame is despite its designation rather a late one too. Its open reading frame is entirely incorporated in E2 and the E4 product is subject to several posttranscriptional modifications. Consequently various E4 protein species can be identified in case of each virus type. The E4 products are involved in the packaging of the viral particles, interacting with the cytoskeleton and with epidermal differentiation (Doorbar, 1991; Bryan, 2000; Roberts, 2002).

Virus life cycle

The target cells of papillomavirus infections are with only few exceptions epithelial cells, specifically epidermal keratinocytes and cells of the mucous membranes. In these tissues, especially in the squamous epithelium, cells undergoing cell division are only present in the basal cell layer. Only these cells are suitable targets for papillomaviruses to establish persisting infections. Several layers of differentiated and differentiating cells however protect these undifferentiated keratinocytes from the hazards of the environment. It is consequently believed that papillomaviruses require sites of small injuries to be able get in contact with those basal cells (Oriel, 1971; Dürst, 1991; zur Hausen 1996).

One of the receptors papillomaviruses attach to is $\alpha 6$ integrin, but it has been shown, that this receptor is not mandatory for all papillomaviruses (Evander, 1997; Sibbet, 2000). The entry mechanism thereafter is clathrin-dependent receptor-mediated endocytosis, at least for most studied papillomaviruses (Day, 2003). Once in the endosome, the L1 of the viral capsid is lost, whereas the L2 mediates the escape into the cytoplasm and later the nuclear localisation of the viral genome (Day, 2004; Kämper, 2006).

After infection there is an initial phase of replication, resulting in a cell containing few to a few hundred copies of the papillomavirus genome. In case of most studied papillomaviruses it is located in episomal form in the nucleus of the infected cell. Some papillomaviruses however, like the human ones HPV16 and HPV18 often integrate into the host genome. This integration is usually associated with a loss of function of the E2 open reading frame and consequently with malignant transformation (Von Knebel, 1988; Desaintes, 1997).

After the early events in the papillomavirus life cycle each genome is copied about once per cell cycle in synchrony with the host genome. This type of maintenance replication is retained in the undifferentiated basal cells (Gilbert, 1987). The differentiation process in the offspring of the infected basal cells however triggers various viral mechanisms like higher E1 and E2 expression. That leads to production of more copies of the genome as well as late gene expression resulting in the production and packaging of viral particles in the apical cells of the tissue (Bedell, 1991; Ozbun, 1998). As papillomaviruses are not lytic viruses, the release of infective viruses is probably due to the normal death of cells in apical layers of epithelia (Lehr, 2003).

Papillomaviruses - pathogens in humans

Although asymptomatic infections are common, various benign and several malignant human skin disorders have been linked to papillomavirus infections (Jablonska, 1997). Cutaneous papillomas like common warts are frequent, usually benign and transient, but in some disorders like epidermodysplasia verruciformis malignancy occurs in rare cases (Jablonska, 1972; Majewski, 1997). Among the variety of known papillomavirus associated cutaneous and mucosal disorders cervical cancer is the best studied and due to its consequences probably the most relevant one. In more than 99% of the cases this disease can be linked to human papillomavirus (HPV) infections (Walboomers, 1999). It has been shown, that HPV infection is mandatory but not sufficient for tumorigenesis in these cases. Several genetic and environmental risk factors have been identified, that may play a role in the development of malignancy which is usually a process of many years. Still, although cofactors play an important role, the type of papillomavirus seems most relevant, as some are found much more frequent in malignant lesions than others. This led to the clinical distinction between high risk and low risk HPVs (Lorincz et al., 1992 and Bosch et al., 1995). High risk HPVs usually have a potential to integrate into the host genome, which is one of the typical mechanisms involved in papillomavirus associated tumorigenesis. The disease complex and its main viral players HPV16 and HPV18 are now best understood ones, even though there are still many open questions (zur Hausen, 1999; zur Hausen, 2000). The intensive research has lead to the development of two prophylactic vaccines against the most abundant HPV types in the past years and future research may enable even better and cheaper vaccines as well as more reliable diagnostic options (zur Hausen, 2002; Gissmann, 2007).

Papillomaviruses in animals

Animals as well as humans are hosts of papillomaviruses and develop benign and malignant lesions as well (Figure 3). Some of these diseases such as warts had actually been known since ancient times, and consequently the first papillomaviruses studied in detail were those inducing neoplasias in cattle, cottontail rabbit and domestic rabbit (Shope, 1933; Parsons, 1943; Black, 1963). After intensive early approaches to uncover the details of papillomavirus biology, which included detailed studies involving the bovine type BPV1 as a model, animal papillomaviruses moved out of the focus of the research community (Chen, 1982; Baker, 1987). Due to the species specificity of most papillomaviruses animals were nevertheless expected to have their own specific papillomaviruses. Still, while about 150 HPV types have yet been entirely and many more partially sequenced papillomaviruses infecting animal species are probably vastly underrepresented. Although to date more than 70 animal papillomaviruses have been entirely sequenced (including those mentioned in this work), only few, a single one or often no papillomaviruses per mammalian species are known (Bernard, 2010; pages 92-93). Only in the past decade, as modern molecular biology methods became more sophisticated and easily available paired with a rising interest in health issues of pets brought again life into this field. It was hypothesised, that in analogy to what is now known about HPVs animals may have various different papillomavirus related disorders, maybe similar to human counterparts (Antonsson, 2002). However, there are still many open questions, not only about the roles of papillomaviruses in animal disease in particular, but also about the evolution and evolutional mechanisms of papillomaviruses in general (Garcia-Vallve, 2005; Gottschling, 2007; Rector, 2007). Even the entire range of hosts is far from being well defined, the fragmentary data nevertheless suggests, that all tetrapods could potentially have their own papillomaviruses (Bernard, 2010).

Image: C. E. Lange, UZH

Figure 2

Canine oral papillomatosis in a young dog.

Aims of the study

While various skin disorder in humans could be linked to a variety of specific HPVs in past decades, papillomatous lesions in animals where often simplistically blamed on "the papillomavirus" as for example canine papillomavirus (CPV1) in dogs. It had been suggested that there might be more than one (Nicholls, 1999), and more recent findings supported that hypothesis (Tanabe, 2000; Narama, 2005; Goldschmidt, 2006; Tobler, 2006; Yuan, 2007). Our studies were consequently aimed to determine whether specific skin disorders may indeed be linked to CPVs other than CPV1. Very closely connected is also the question whether the hypothesis that each animal might harbour an own set of papillomaviruses is true or not, as there was evidence for this in cattle (Campo, 1984; Hatama, 2008; Hatama 2009). To determine the relevance of putative papillomavirus findings it was furthermore of interest whether CPV DNA like HPV DNA may be present on the skin in the absence of lesions as well (Antonsson, 2000; Antonsson, 2003). To learn more about cellular mechanisms of papillomavirus infections in general and in dogs in particular, establishing a cell culture based system to study the effects of CPVs on their natural host cells was also aspired.

A second focus was on equine papillomaviruses (EcPVs). Horses are frequently affected by papillomavirus associated lesions; however, most of the knowledge about papillomaviruses in horses is connected to the bovine papillomaviruses BPV1 and BPV2 which induce the equine sarcoid (Scott, 2003; Nasir, 2008). We intended to figure out whether other papillomatous equine disorders may be linked to papillomavirus infection and if so to which papillomaviruses (Scott, 2003).

Thesis structure

This thesis is divided into two parts. The first part focuses on different aspects of canine papillomaviruses and papillomatoses and the second part on equine papillomaviruses.

Four manuscripts, three of them presented in their published layout and one in a publication ready format, make up the first part. The first manuscript deals with the question whether certain disorders of dogs are associated with papillomaviruses, and if so with which ones. Based partially on the results from the first article the second manuscript deals with the genetic and phylogenetic characterisation of the seven canine papillomaviruses. While these two address questions of association, the third article focuses on screening methods and the counter question whether papillomavirus DNA can also be found on clinically healthy skin. The part about canine papillomaviruses concludes with a manuscript describing the generation of a cell culture model for the study of papillomavirus cell interactions.

Two manuscripts, one already published and one submitted, are combined to form the second part which is devoted to equine papillomaviruses. The first manuscript introduces two novel equine papillomaviruses and places them into a clinical and phylogenetic context. The second focuses on one of the virus associated disorders and seeks to support the hypothesis of causality using different methods.

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Part I – Canine papillomaviruses and papillomatoses in dogs

Although the existence and the transmissive character of papillomatoses in dogs has been known for a long time the knowledge about the involved viruses and the molecular mechanisms remained rather fragmentary. The best studied canine papillomavirus (CPV) is the one inducing the canine oral papillomatosis CPV1 (prior known as COPV). After CPVs 2 and 3 had been discovered a few years ago we intended to determine whether the three CPVs may be sufficient to explain the different pathologies in dogs and to develop a culture model. We identified four additional CPVs associated with various pathologies; three of those were cloned and sequenced. We found, that histologically different types of inverted papillomas seem to be associated with the DNA of different CPVs (*Canine inverted papillomas associated with DNA of four different papillomaviruses, pages 15-20*). The analysis of the genomic sequences of the newly cloned viruses revealed that they could be allocated to the same three genera as CPVs 1, 2 and 3, namely papillomavirus genera Lambda, Tau and Chi. This phylogenetic characterization was supported by other genomic features like for example genome organization and genome size. Also some shared clinical-pathological characteristics were reflected in this categorization, most evident is that the Chi genus harbours only CPV associated with pigmented plaques or else all CPVs inducing pigmented plaques are Chi papillomaviruses (*Three novel canine papillomaviruses support taxonomic clade formation, pages 21-30*). Eight broad range PCRs with published primer combinations were thoroughly evaluated for future research and diagnostic purposes in dogs. Based on these results the question whether dogs like humans may harbour papillomaviral DNA on their clinically health skin was addressed. We detected CPV DNA in many samples of oral mucosa and interdigital skin, thus confirming the hypothesis (*The clinically healthy skin of dogs is a potential reservoir for canine papillomaviruses, pages 31-37*). To have a tool for future research on the mechanisms of virus cell interaction the generation of a cell culture model was approached. Canine keratinocyte cell lines were successfully transduced using the amplicon system to harbour and translate the DNA of CPV1, CPV3 or CPV5 respectively (*Generation of canine keratinocyte cell lines stably harbouring canine papillomavirus DNA, pages 38-52*).

Canine inverted papillomas associated with DNA of four different papillomaviruses

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Own contribution

I was substantially involved in the planning and outlining of this work. I performed all the molecular biological experiments and a major part of the data analysis. I also did the major part of the writing.

In detail, figures: Figures 2 and 6 by C. E. Lange, figure 1 by W. v. Bomhard, figures 3 and 5 by K. Brandes, figure 4 by L. Ordeix

In detail, text: Abstract by C. E. Lange, C. Favrot and K Tobler, introduction by C. Favrot and C. E. Lange, material and methods by C. E. Lange, K. Tobler and C. Favrot, results by all authors, discussion by C. E. Lange, C. Favrot and K. Tobler

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Canine inverted papillomas associated with DNA of four different papillomaviruses

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Abstract

Inverted papillomas are uncommon papillomavirus (PV)-induced canine skin lesions. They consist of cup- to dome-shaped dermal nodules with a central pore filled with keratin. Histologically they are characterized by endophytic projections of the epidermis extending into dermis. Cytopathic effects of PVs infection include the presence of clumped keratohyalin granules, koilocytes and intranuclear inclusion bodies. Different DNA hybridization studies carried out with a canine oral papillomavirus (COPV) probe suggested that a different PV than COPV might cause these lesions. Canine papillomavirus 2 (CPV2) was discovered a few years ago in inverted papillomas of immunosuppressed beagles. Two other cases, presenting with distinct clinical and histological features have also been described. This study was carried out on four dogs with clinical and histological signs of inverted papillomas. Molecular biological analyses confirmed that PV DNA was present in all four lesions but demonstrated that the sequences in each case were different. One corresponded to COPV, the second to CPV2, and the third and fourth to unknown PVs. These findings suggest that inverted papillomas are not caused by one single PV type. Similar observations have also been made in human medicine.

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Introduction

In dogs papillomaviruses (PVs) induce several skin changes including exophytic papillomas, pigmented plaques and inverted papillomas. The last are rather uncommon PV-induced canine skin lesions that consist of cup- to dome-shaped nodules with a central pore filled with keratin.[1] Four different subtypes regarding body location, shape, size and colour have been reported. Classical inverted papillomas consist of large (1–2 cm) cup-shaped, greyish nodules with a large central pore and were initially described by Campbell *et al.*[2] Affected animals usually present with one or few lesions on the abdomen. The second subtype was described by Shimada *et al.* and consists of smaller (4 mm) dome-shaped flesh-coloured lesions disseminated all over the body.[3] Le Net *et al.* described a few years ago very small (2 mm) disseminated black papules with particular histological features (see below: intracytoplasmic eosinophilic inclusions) in a Boxer.[4] Finally, inverted papillomas were observed concomitantly with classical exophytic papillomas by Goldschmidt *et al.* in immunosuppressed beagles, most of them developing interdigitally.[5]

From the histological point of view, all inverted papillomas consist of endophytic, papillary projections of the epidermis extending into dermis. The cytopathic effects of PV infection include presence of clumped keratohyalin granules, koilocytes and less frequently, basophilic or eosinophilic intranuclear inclusions.[2–6] The papillomas described by Le Net stand out from the others because of their large, eosinophilic intracytoplasmic inclusions.[4]

Immunohistochemistry was used to confirm that PVs replicate in inverted papilloma lesions and, consequently, probably play a major role in their development. DNA hybridization studies carried out with a canine oral papillomavirus (COPV) probe suggested however that these lesions may be due to a different PV from that causing classical canine oral warts.[2] In fact, canine papillomavirus 2 (CPV2) was identified a few years ago in inverted papillomas from immunosuppressed Beagles.[5,7] All available clinical, histopathological and virological information suggest that inverted papillomas are probably not caused by one single virus.

This study was undertaken on four dogs with inverted papillomas, each representing one example of the four phenotypes described above. Clinical, histological and

virological features of these lesions are presented. Molecular biological analyses were carried out with combinations of PCR primers designed to amplify as many canine papillomavirus as possible. The goal of the study was to demonstrate the genetic diversity of canine inverted papilloma-associated PVs.

Materials and methods

Clinical and histological features

Four dogs with cutaneous nodules resembling inverted papillomas were included. Lesions were surgically removed and histological examination was carried out, using standard methods.

Molecular biological analyses

DNA was extracted from paraffin embedded tissues (cases 1, 2 and 4) or frozen tissue (case 3) using a DNeasy extraction kit (Qiagen, Basel, Switzerland). To identify PV DNA a combination of primers optimised for CPV detection was used, consisting of FAP64 and canPVf (CTT CCT GAW CCT AAY MAK TTT GC).[8] Amplification was undertaken in a PTC-200 thermo cycler (MJ Research, Watertown, MA, USA) under conditions of 94°C for 10 min followed by 45 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. A final extension of 72°C for 10 min concluded the PCR. Electrophoresis in a 1% agarose gel containing ethidium bromide was used to detect the amplified fragments. The amplified sequences deriving from the L1 open reading frame were extracted from the gels with a QIAEX II (Qiagen) kit and were determined commercially (Microsynth) by cycle sequencing using an ABI 377 sequencer (Applied Biosystems). The obtained sequences of both strands were aligned and only the central double sequenced region excluding primer sequences and inconclusive end/start regions were used for further analysis (GQ204117–GQ204120). These sequences were compared with the NCBI gene bank using BlastX and BlastN (February 2009) analysis. Amino-acid alignments were performed in CLUSTAL_X version 1.83 with the default parameters; the output data were edited in Word (Microsoft).[9]

Results

Animals and clinical features

A 5-month-old male Flat coated Retriever (Dog 1) with an erythematous otitis externa and an 8 mm diameter greyish nodule on the abdomen (Figure 1) that was surgically removed and did not recur.

Figure 1. Case 1: Large classical 'Campbell-type' inverted papilloma on the abdomen. Note the typical dome-shape aspect and the central pore.

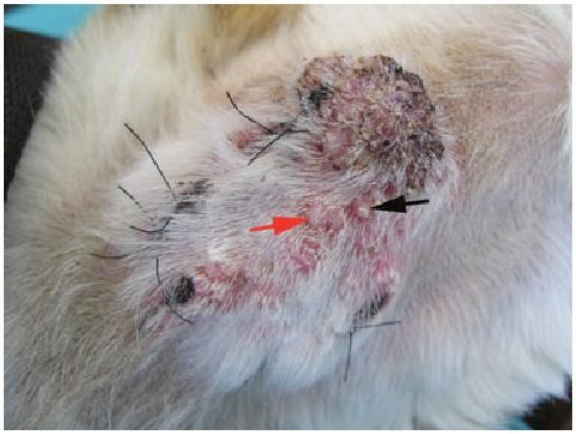

Figure 2. Case 2: Smaller 'Shimada-type' inverted papillomas. Note the dome-shaped aspect (red arrow) and the keratin plugs (black arrow).

A 11-year-old intact female Beagle (Dog 2) with an 8 mm diameter greyish nodule on the paw pad that never recurred following surgical removal.

A 4-year-old female Beagle (Dog 3) with a pruritic plaque on the ventral neck, evolving concomitantly with a pyometra. The skin lesion consisted of numerous small (1–2 mm diameter) dome-shaped white papular lesions (Figure 2). The dog was spayed and skin samples were taken by biopsy for histopathological and virological examination. The lesions regressed spontaneously within 30 days.

The fourth case was a 5-year-old female Rhodesian Ridgeback (Dog 4) with four black small nodules (2 mm diameter).

Lesions were surgically removed: three were melanocytomas and one (concave face of the pinna) an inverted papilloma. Lesions did not recur after excision.

Histpathological features

All the papillomas shared a sharply demarcated cup-shaped aspect characterized by centripetal papillary projections of hyperplastic squamous epithelium with a central core of keratin layers including parakeratotic cells (Figure 3) although the lesions in dogs 3 and 4 were smaller. The basal layers were hyperplastic with a moderate number of mitotic figures. Multifocally, the subcorneal epithelium showed a variable number of koilocytes occasionally with basophilic intranuclear inclusions and a few large keratohyalin granules (Figure 4). Additionally, in one case (case 4), eosinophilic intracytoplasmic pseudoinclusions were observed (Figure 5) while papillary projections were moderate. Based on these observations a diagnosis of inverted papilloma was made.

Molecular biological analyses

The histological findings of koilocytes and intranuclear inclusions suggest the replication of PVs in the four cases. The PV specific DNA amplification and PCR sequencing in each of the four samples revealed in case 1 that the sequence obtained (GQ204117) corresponded at the amino acid level (BlastX) exactly to that of COPV (97/97). In case 2 the sequence obtained (GQ204118)

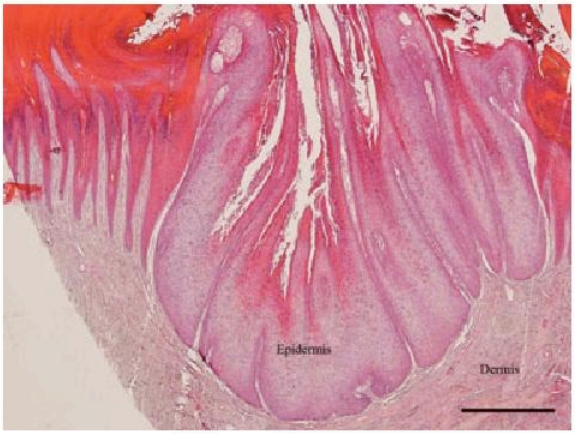

Figure 3. Case 2: Microscopic appearance of an inverted papilloma. Note the papillary projections in the dermis of the squamous epithelium. H&E. Bar = 0.5 mm.

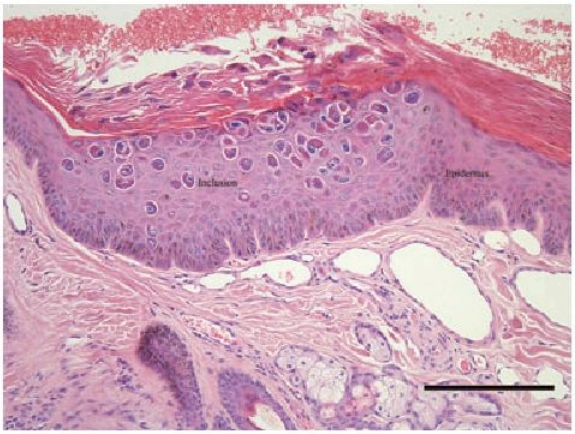

Figure 5. 'LeNet-type' papilloma. Note the large intracytoplasmic eosinophilic inclusions (I) in the epidermis (E). H&E. Bar = 50 μm.

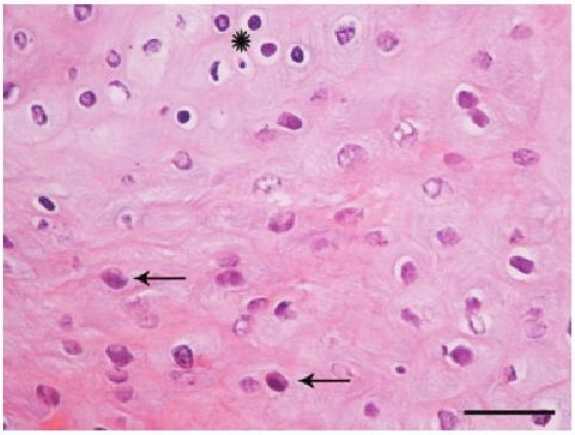

Figure 4. Inverted papilloma (case 2). Note the presence of koilocytes (star) and intranuclear inclusions (arrows) in the lesional epidermis. H&E. Bar = 50 μm.

coded for exactly the same amino acids as CPV2 (94/94).[5,7] The analysis of the third sample revealed a sequence (GQ204119) not identical with any published PV sequence. The highest similarities with this sequence were found among members of the genus Lambda PV. According to the translated sequence COPV was identified as the closest canine PV sharing 70% identity (68/96). Identity was highest among the Lambda PVs of the cat (FdPV1), the lynx (LrPV1), the puma (PcPV1) with 78% each (75/96) and the snow leopard (UuPV1) with 75% (72/96). In case 4 another thus far unknown PV DNA (GQ204120) was uncovered. The BlastX comparison revealed again similarities with Lambda PVs and COPV being the closest canine PV with 70% identity (67/95). Also PcPV1 with 71% (69/96), UuPV1 (67/95) and LrPV1 (68/96) with 70% each displayed similarity to the novel sequence. The homologies and differences of the four sequences were also analysed based on an alignment of an 84 amino-acid stretch covered by all four sequences (Figure 6). It points out, that aligned amino acid sequences of cases 1 and 2 are identical to references sequences of COPV and CPV2, respectively. In contrast, cases 3 and 4 sequences do not match any known, PV sequence. The two DNAs amplified in samples three and four were additionally analysed at the nucleotide level (BlastN), and were found to share 74% identities (212/284).

According to these results each of the four unique cases was associated with a different PV, two of which are as yet unknown.

Discussion

Canine endophytic (inverted) papillomas have been reported only infrequently.[1–6,10] PV-specific cytopathic changes were observed in all reported cases. The latter changes, as well as immunostaining studies support the causative role of PV(s).[2,3,6] Our results demonstrating the presence of PV DNA suggest that these viruses may be the causative agent of these lesions and the phenotypic

```
          98                                                                  180
COPV      RLVWGLRGLEIGRGQPLGISVTGHPTFDRYNDVENPNKNLAGEGGG-TDSRVNMGLDPKQTQMFMIGCKPALGEHWSLTRWCTG
Case 1    RLVWGLRGLEIGRGQPLGISVTGHPTFDRYNDVENPNKNLAGHGGG-TDSRVNMGLDPKQTQMFMIGCKPALGEHWSLTRWCTG
Case 3    RLVWALRGLEIDRGQPLGVSVTGNTTFDRYSDVENPNKNPTDHDKENTDPRVNVALDPKQTQLFLVGCKPAIGEHWIQARWCVG
Case 4    RLVWGLRGLEIDRGQPLGISVTGNPTFDKFSDVENSNKVQTDHDKD-ADTRVNIGLDPKQTQLFLIGCKPAIGEHWVQARWCVG
Case 2    RLVWRLTGIEIGRGGPLGFGTTGNFLFDRLQDTENPNNTKVAT----TDDRQNVSMDPKQTQLFVVGCTPCKGEHWDQAPRCDN
CPV2      RLVWRLTGIEIGRGGPLGFGTTGNFLFDRLQDTENPNNTKVAT----TDDRQNVSMDPKQTQLFVVGCTPCKGEHWDQAPRCDN
          96                                                                  175
```

Figure 6. Alignment of the overlapping putative amino-acid (AA) sequences encoded by the PCR fragments. Reference sequences of COPV (NC_001619) and CPV2 (NC_006564) in bold. Conserved AAs are underlaid in gray. Numbers indicate first and last amino acid of the reference L1 proteins. Note that the sequence corresponding to case 1 is identical to COPV and the sequence corresponding to case 2 identical to CPV2, while the sequences associated with cases 3 and 4 exhibit various differences to the presented sequences.

differences support reports in the literature that suggest that more than one PV may be involved. The cases in this study illustrated this phenotypic variability: In fact, case 1 was very similar to the description made by Campbell, while cases 2, 3 and 4 likely correspond to the Goldschmidt-, Shimada- and Le Net-types, respectively.[2–5] The PV DNA sequences demonstrated clearly the presence of four different viruses but the finding of these PV DNAs in the lesions does not prove causality as PV DNA has been demonstrated on unaffected skin in several other species.[11–13] However, the histological findings clearly indicate viral replication and causality is consequently likely. Interestingly the four papillomas were harbouring the DNA of one unique PV each. Hints for the presence of more than one PV DNA in each lesion were not present, but cannot entirely be ruled out due to the limited knowledge about the genetic diversity of canine PVs. The DNA found in case 1 matched exactly with COPV, which is known to induce oral papillomas in canids but has also been found in other papillomas. This result contrasts with DNA hybridization studies carried out by Campbell *et al.*, which excluded the presence of COPV in similar lesions.[2] In contrast, CPV2 DNA was amplified from case 2, which shares many similarities with cases described by Goldschmidt *et al.*[5,7] The viral DNA found in cases 3 and 4 appears to belong to two novel and distinct canine PVs but the sequences suggest, that these two viruses may belong to the same genus as COPV (Lambda PVs).

Inverted papillomas are infrequently observed in humans.[14] Most of them occur in the nasal cavity, although cutaneous involvement has been described.[15] Several PVs have been associated with the development of these lesions and neoplastic transformation often occurs when high-risk human PVs are involved.[14,16,17]

From the four cases presented here it can be speculated, that in dogs as in humans certain PVs might be associated with distinct forms of endophytic, inverted papillomas. Further studies are required to confirm the genetic diversity of inverted papillomas-associated PVs and to better characterized the novel PVs.

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Résumé Les papillomes inversés sont des lésions cutanées canines rares, liées à un papillomavirus (PV). Ils consistent en des nodules dermiques convexes ou en dôme avec un pore central rempli de kératine. Histologiquement, ils sont caractérisés par des projections endophytiques de l'épiderme jusque dans le derme. Les effets cytopathogènes d'une infection à PV incluent la présence de granules agglomérées de kératohyaline, de koïlocytes et de corps d'inclusion intranucléaires. Différentes études d'hybridation d'ADN effectuées à partir de papillomavirus canin oral (COPV) ont montré qu'un PV différent de COPV pourrait être à l'origine de ces lésions. Le papillomavirus canin 2 (CPV2) a été découvert il y a quelques années dans les papillomes inversés de beagles immunodéprimés. Deux autres cas, présentant des caractéristiques cliniques et histologiques distinctes ont également été décrits. Quatre chiens présentant des signes cliniques et histologiques de papillomes inversés ont été inclus dans cette étude. Les analyses biologiques moléculaires ont confirmées que l'ADN de papillomavirus était présent dans les quatre lésions mais les séquences étaient à chaque fois différentes. L'une correspondait à COPV, la seconde à CPV2, la troisième et la quatrième à des PV non décrits. Ces résultats suggèrent que les papillomes inversés ne

sont pas dus à un seul type de PV. Des observations identiques ont également été faites en médecine humaine.

Resumen Los papilomas invertidos con lesiones de la piel poco comunes inducidas por el virus papiloma (PV). Consisten en nódulos dérmicos en forma de copa o cúpula con un poro central relleno de queratina. Histológicamente se caracterizan por proyecciones endofíticas de la epidermis que se extienden en la dermis. Los efectos citopáticos de la infección con PV incluyen la presencia de gránulos de queratohialina agrupados, koilocitos y cuerpos de inclusión intranucleares. Estudios de hibridación de DNA llevados a cabo con un segmento de papilomavirus canino oral (COPV) indican que un virus diferente puede ser el causante de la lesiones. Hace unos años se caracterizó el papilomavirus canino tipo 2 en papilomas invertidos de perros Beagle inmunosuprimidos. Otros dos casos con características clínicas e histológicas diferentes también han sido descritos. Este estudio se desarrollo en cuatro perros con signos clínicos de papilomas invertidos confirmado por histopatología. Un análisis moléculas confirmó la presencia de DNA de PV en las cuatro lesiones pero indicó que la secuencia era diferente en cada lesión. Uno correspondía a COPV, el segundo a CPV2 y el tercero y el cuarto a PV desconocidos. Estos hallazgos sugieren que los papilomas invertidos no están causados por un solo tipo de PV. Observaciones similares se han realizado en medicina humana.

Zusammenfassung Invertierte Papillome sind unübliche Papillomavirus (PV)-induzierte canine Hautveränderungen. Sie bestehen aus becher- bis kuppelförmigen dermalen Knoten mit einer zentralen Pore, die mit Keratin gefüllt ist. Histologisch werden sie charakterisiert durch endophytische Projektionen der Epidermis, die sich bis in die Dermis erstrecken. Zu den zytopathischen Auswirkungen der PV-Infektion zählen das Auftreten von geklumpten Keratohyalingranula, Koilozyten und intranukleäre Einschlusskörperchen. Verschiedene DNA Hybridisierungsstudien, die mit einer canines oralen Papillomavirus (COPV) Sonde durchgeführt wurden, zeigten Hinweise darauf, dass ein anderer PV als das COPV die Ursache für diese Läsionen sein könnte. Der canine Papillomavirus 2 (CPV2) wurde vor einigen Jahren in invertierten Papillomen von immunsupprimierten Beagles entdeckt. Zwei weitere Fälle, die sich mit eindeutigen klinischen und histologischen Charakteristika präsentierten, sind ebenfalls beschrieben. Diese Studie wurde an vier Hunden mit klinischen und histologischen Anzeichen von invertierten Papillomen durchgeführt. Eine molekularbiologische Analyse bestätigte, dass PV DNA in allen vier Veränderungen vorhanden war, zeigte allerdings, dass die Sequenzen für jeden Fall verschieden waren. Eine stimmte mit der von COPV überein, die zweite mit CPV2 und die dritte und vierte mit unbekannten PVs. Diese Ergebnisse sind ein Hinweis darauf, dass invertierte Papillome nicht von einem einzigen PV Typ verursacht werden. Ähnliche Beobachtungen wurden auch in der Humanmedizin gemacht.

Three novel canine papillomaviruses support taxonomic clade formation

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Own contribution

I did most of the planning and outlining of this work. I performed all the molecular biological experiments and a major part of the data analysis. I also did the major part of the writing.

In detail, figures and tables: Figures 1 and 2 by C. E. Lange and K. Tobler, tables 1 and 3 by C. E. Lange, table 2 by C. E. Lange and K. Tobler, table S1and figure S1 by C. E. Lange

In detail, text: Abstract by C. E. Lange and K. Tobler, introduction by C. E. Lange, methods by all authors, results by C. E. Lange and K. Tobler, discussion by C. E. Lange, C. Favrot and K. Tobler

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Three novel canine papillomaviruses support taxonomic clade formation

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More than 100 human papillomaviruses (HPVs) have been identified and had their whole genomes sequenced. Most of these HPVs can be classified into three distinct genera, the alpha-, beta- and gamma-papillomaviruses (PVs). Of note, only one or a small number of PVs have been identified for each individual animal species. However, four canine PVs (CPVs) (COPV, CPV2, CPV3 and CPV4) have been described and their entire genomic sequences have been published. Based on their sequence similarities, they belong to three distinct clades. In the present study, circular viral DNA was amplified from three dogs showing signs of pigmented plaques, endophytic papilloma or *in situ* squamous cell carcinoma. Analysis of the DNA sequences suggested that these are three novel viruses (CPV5, CPV6 and CPV7) whose genomes comprise all the conserved sequence elements of known PVs. The genomes of these seven CPVs were compared in order properly classify them. Interestingly, phylogenetic analyses, as well as pairwise sequence alignments of the putative amino acid sequences, revealed that CPV5 grouped well with CPV3 and CPV4, whereas CPV7 grouped with CPV2 but neither group fitted with other classified PVs. However, CPV6 grouped with COPV, a lambda-PV. Based on this evidence, allocation of CPVs into three distinct clades could therefore be supported. Thus, similar to HPVs, it might be that the known and currently unknown CPVs are related and form just a few clades or genera.

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INTRODUCTION

Papillomaviruses (PVs) are small, non-enveloped, double-stranded DNA viruses that have a tropism for the skin and mucosal membranes (Howley & Lowy, 2007). They have been found to be innocuous inhabitants of the healthy skin but are also associated with various neoplastic diseases (Howley & Lowy, 2007). Several PV types have a high oncogenic potential; others are known to be less oncogenic, facultatively pathogenic or thought to be apathogenic (Antonsson *et al.*, 2000; Antonsson & Hansson, 2002; Burd, 2003; Munoz *et al.*, 2003).

Categorization of the PVs based on their oncogenic potential has been suggested, grouping the known viruses into high-, intermediate- and low-risk PVs (Lorincz *et al.*, 1992; Bosch *et al.*, 1995). This grouping is only partly reflected in the genomic characterization of the PVs. The viruses combined in one genus based on the nucleotide sequence of the L1 open reading frame (ORF) may share very distinct features, but the biological effects of these can differ significantly (de Villiers *et al.*, 2004). Knowledge of the phylogenetics, the genomic organization and the biological effects taken together may be the best way of characterizing PVs.

So far more than 100 human PVs (HPVs) have been isolated, cloned and sequenced completely and many more have been partially sequenced. They group into the genera alpha-, beta-, gamma-, mu- and nu-PV. Not only HPVs but also a growing number of animal PVs have been detected in lesional and healthy skin of mammals, birds and even reptiles in recent years. It would be anticipated that, in addition to humans, each animal species might carry a large set of PV types. So far, only one or a very small number of PVs have been completely sequenced for most animal species. In very few animal species, viruses belonging to different genera have been identified.

The GenBank accession numbers for the sequences reported in this paper are FJ492742–FJ492744.

A supplementary figure and a supplementary table are available with the online version of this paper.

Categorization of these frequently single standing viruses is often difficult or impossible at this time, as close relatives are usually missing.

A total of four virus genomes obtained from dogs have been published to date (Delius *et al.*, 1994; Tobler *et al.*, 2006, 2008; Yuan *et al.*, 2007). These seem to belong to three different clades of which only one (lambda-PV) has been named. The possible relevance of these clades is not yet clear and their characteristics are, at present, vague.

In this study, we sequenced the DNA of three new canine papillomaviruses (CPVs) extracted from three clinically affected dogs, one of them suffering from pigmented plaques, one from an inverted papilloma and one from an *in situ* squamous cell carcinoma (SCC). Rolling circle amplification, restriction enzyme analysis, genomic cloning and primer-walking sequencing were applied. The new viruses CPV5, CPV6 and CPV7 were compared with the other known CPVs in terms of their genomes and genomic features, their phylogenetics and the corresponding lesions. A clear grouping of the new viruses into the three proposed clades was possible and some additional characteristics could also be identified.

METHODS

Animals and samples. CPV samples were taken from three dogs. Dog one was presented at a small animal hospital in Birkenfeld, Germany, with numerous pigmented and partially hyperkeratotic macules, papules, patches and nodules distributed over the whole body. Dog two was presented at the veterinary teaching hospital in Zurich, Switzerland, with a large plaque on the ventral throat. This plaque consisted of numerous dome-shaped papules with a central plug of keratin. Dog three was a 5-year-old male trailhound presented at the veterinary teaching hospital in Edinburgh, Scotland, with hyperkeratotic plaques on the concave faces of the pinnae.

In order to make a diagnosis, 6 mm skin biopsies were taken and sent to three different histopathology laboratories, where they were processed routinely (haematoxylin and eosin staining). Additional samples were frozen immediately after excision and were sent to the Dermatology Unit of the Vetsuisse Faculty in Zurich, Switzerland, for virological analysis. Histological analyses revealed that the samples from dogs one, two and three corresponded to canine pigmented plaques, inverted papillomas and *in situ* SCC, respectively.

Genome amplification and cloning. Total DNA from 25 mg tissue samples of the three dogs was isolated by using a DNeasy extraction kit (Qiagen) according to the manufacturer's recommendations. DNA (1 µl) was used for rolling circle amplification (Rector *et al.*, 2004), using a TempliPhi Amplification kit (General Electrics Biosciences). The protocol supplied by the manufacturer was used, with slight modifications: 1 µl of 10 mM dNTPs was added and the reaction time was prolonged to 16 h at 30 °C. Amplified DNA was digested with different restriction enzymes to compare the restriction patterns and to identify possible single cutters. Amplified DNA was cloned into pBluescript II-KS$^+$ (Stratagene) using standard procedures. Initially, the *Xba*I and *Cla*I sites, the *Eag*I and *Spe*I sites and the *Hin*dIII and *Bam*HI sites were used in cases one, two and three, respectively. The *Cla*I, *Eag*I and *Hin*dIII clones, respectively, were identified as containing the complete genomes.

Sequence analysis. The nucleotide sequence of cloned DNA and precipitated rolling circle amplification product was determined commercially (Microsynth) by cycle sequencing using an ABI 377 sequencer (Applied Biosystems). The primary sequences were assembled using Contigexpress (Vector NTI Informax). Besides the CPVs, 43 PVs covering all genera and including various unclassified PVs were chosen for the pylogenetic analysis. Some PVs associated with cutaneous lesions in humans were also included. Multiple sequence alignments of the L1 protein sequences were done with Mafft [version 6.611b: Blosum 62 matrix (Katoh & Toh, 2008)]. Aligned cDNA sequences were produced by aligning the coding sequences with the corresponding aligned amino acid sequences. Bayesian phylogeny was estimated with MrBayes (version 3.1.2: Markov Chain Monte Carlo (MCMC) with GTR substitution matrix, variable gamma rates, two runs with four chains of 1 000 000 generations; Ronquist & Huelsenbeck, 2003). Evaluation of the MCMC and final refinements of the tree were performed with BEAST (version 1.4.8; Drummond & Rambaut, 2007).

RESULTS

Genomes and ORFs

PV genomes are characteristically approximately 8 kb and have circular genomic organization usually containing six to eight ORFs as well as one or two non-coding-regions (NCRs) (Howley & Lowy, 2007). To compare the genomes of the seven CPVs, they were all annotated in the same way. The genome sizes of the CPVs vary by more than 800 bp (Fig. 1), ranging from 7742 bp (CPV4) to 8607 bp (COPV), which is still the largest described PV genome. A comparative analysis of alpha-, beta- and gamma-HPV genome sizes showed a significant correlation between genome length and genus (Supplementary Fig. S1, available in JGV Online). In all CPV genomes, seven characteristic ORFs can be identified (Fig. 1). While the ORFs of the late genes appear to be quite conserved in terms of size, certain differences can be observed among the early genes and the NCRs (Table 1). Most prominent is the typical lambda-PV pattern with a very large second NCR (NCR2) and rather small E2 and E4 ORFs; this pattern is present in COPV and CPV6. In the case of CPV3, CPV4 and CPV5, the NCR2 is approximately 200 nt smaller, while the NCR2 of both COPV and CPV6 is at least twice as long. According to the genome size and organization, the viruses might be grouped as follows: COPV and CPV6; CPV2 and CPV7; and CPV3, CPV4 and CPV5.

Characteristic motifs

Besides the ORFs, several characteristic features that are predicted to play a role in the PV life cycle are known at the nucleotide and amino acid level (Androphy *et al.*, 1987; Howley & Lowy, 2007; Münger *et al.*, 2004; Wilson *et al.*, 2002). All CPV sequences were scanned to identify these putative sites and the analysis revealed typical features in the genomes of all seven sequences (Supplementary Table S1, available in JGV Online). The GC content of the entire genomes is 40–53 %. The clade comprising CPV3, CPV4 and CPV5 shows the highest GC content, with 51, 53 and

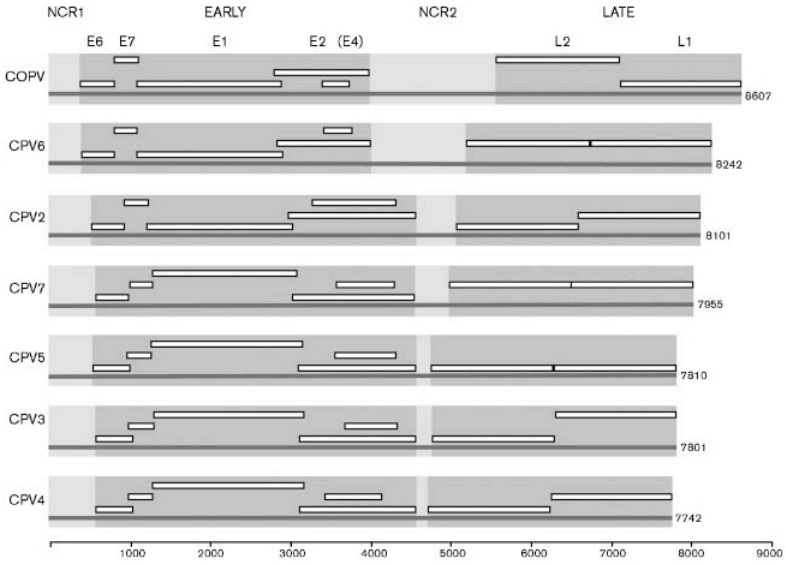

Fig. 1. Schematic presentation of the CPV genomes and ORFs. The genomes are divided into three sections: early genes (EARLY), late genes (LATE) and non-coding regions (NCR1 and -2). Numbers indicate nucleotide positions. Nt 1 is set as the first following the stop codon of the L1 ORF.

50 %, respectively. Besides transcription factor binding sites for SP1 and NF1 and the TATA signals, the sequence elements for PV DNA replication were found in all seven viral genomes. In particular, a dyad symmetry repeat flanked by two E2 binding sites is present on each genomic sequence within the NCR1. Examination of the putative protein sequences reveals an ATP-dependent helicase motif located in each E1 and two and one metal-binding motifs in E6 and E7, respectively.

Sequence analysis and comparison

In order to evaluate the possible classification of the three newly recognized canine PVs, phylogeny based on the L1 sequences was determined. Amino acid sequences of 50 L1 proteins, including those from the three newly recognized canine PVs, were aligned; the alignment was then back-translated into the nucleotide sequences and used to infer the phylogeny of the 50 PVs (Fig. 2). Based on the resulting tree, CPV3, CPV4 and CPV5 are located on one clade of the tree close to bovine PV (BPV)-1 and ovine PV-1 (delta-PV) and BPV5 (epsilon-PV) and *Equus caballus* PV1 (zeta-PV). Likewise, CPV7 neighbours CPV2 and is in proximity to HPV4 and HPV50 (gamma-PV), and CPV6 neighbours *Felis domesticus* PV1 and COPV (lambda-PV). In conclusion, each of the three newly recognized canine PVs is related to one previously described canine PV.

In order to investigate the relatedness of the canine PVs further, pairwise alignments were performed for each individual protein sequence of each virus against all other viruses (Table 2). The most conserved amino acid sequences are L1 and E1. Other protein sequences, such as E2 (24–65 %) and L2 (29–78 %), are generally less conserved among the CPVs. The intra-clade comparisons of the E1 proteins revealed 65–81 % identity, whereas the inter-clade revealed only around 40 % (39–44 %) identity. Similarly, intra-clade comparisons of the L1 proteins revealed 70–82 % identity, whereas the inter-clade revealed

Table 1. Size and position of predicted ORFs and NCRs

Sizes are given in bp.

ORF	COPV	CPV2	CPV3	CPV4	CPV5	CPV6	CPV7
E6	432 (364–795)	405 (496–900)	453 (570–1022)	453 (560–1012)	456 (563–1018)	405 (388–792)	414 (488–901)
E7	291 (795–1085)	294 (906–1199)	315 (970–1284)	297 (975–1271)	300 (981–1280)	291 (792–1082)	294 (906–1199)
E1	1791 (1078–2868)	1821 (1186–3006)	1884 (1277–3160)	1887 (1264–3150)	1887 (1273–3159)	1791 (1075–2883)	1815 (1186–3000)
E2	1191 (2777–3967)	1593 (2948–4540)	1449 (3105–4553)	1461 (3095–4555)	1467 (3104–4570)	1191 (2828–3991)	1512 (2842–4453)
E4	348 (3378–3725)	1047 (3249–4295)	645 (3670–4314)	705 (3423–4127)	762 (3570–4331)	348 (3405–3755)	732 (3480–4211)
L2	1539 (5550–7088)	1524 (5053–6576)	1515 (4764–6278)	1524 (4700–6223)	1515 (4769–6283)	1539 (5192–6724)	1530 (4893–6422)
L1	1509 (7099–8607)	1509 (6593–8101)	1500 (6302–7801)	1497 (6246–7742)	1503 (6308–7810)	1509 (6737–8242)	1512 (6444–7955)
NCR1	363 (1–363)	495 (1–495)	569 (1–569)	559 (1–559)	562 (1–562)	387 (1–387)	487 (1–487)
NCR2	1582 (3968–5549)	512 (4541–5052)	210 (4554–4763)	144 (4556–4699)	198 (4571–4768)	1200 (3992–5191)	439 (4454–4892)

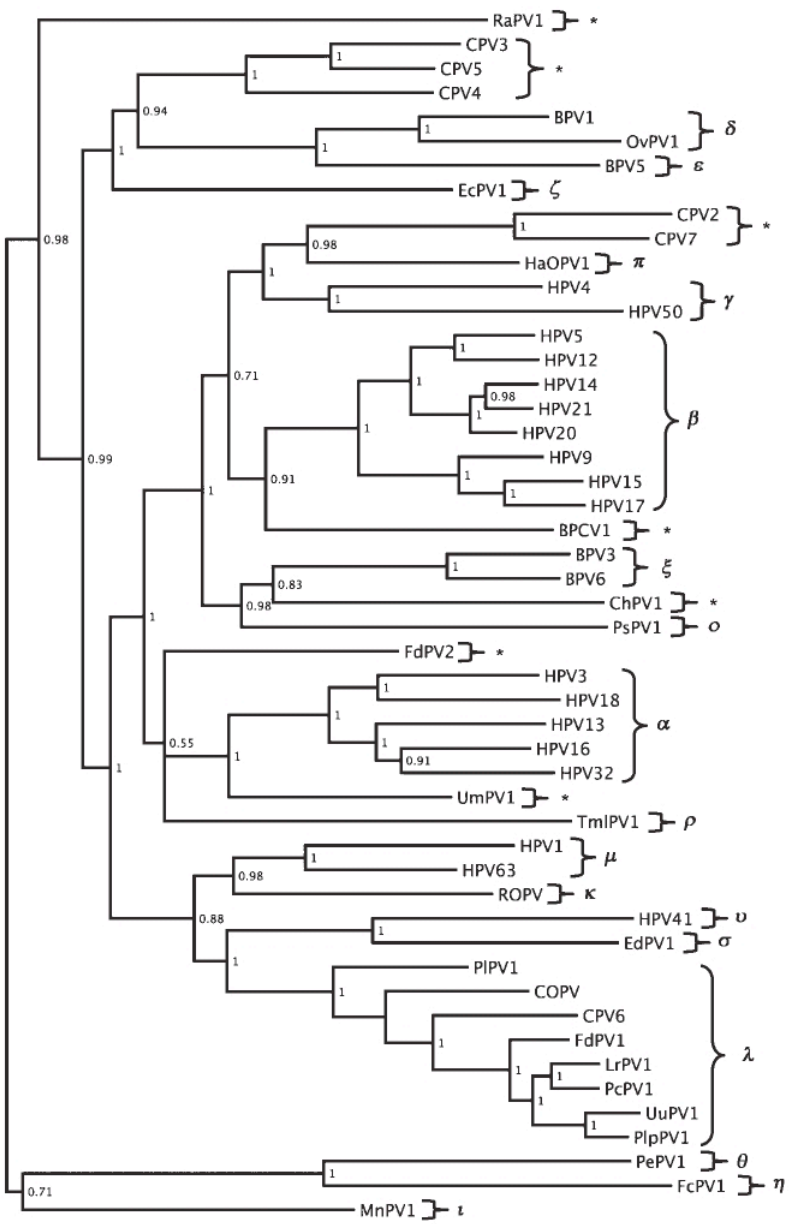

Fig. 2. Neighbour-joining phylogenetic tree of the L1 nucleotide sequences of 50 PVs. The PV genus of each strain is indicated. PVs and/or putative PV genera that are currently unclassified are marked with an asterisk. The PVs (with their GenBank accession nos) are: BPV1 (NC_001522), BPV3 (NC_004197), BPV5 (NC_004195), BPV6 (AJ620208); bandicoot papillomatosis carcinomatosis PV (BPCV1) (NC_010107); COPV (NC_001619); CPV2 (NC_006564), CPV3 (NC_008297), CPV4 (NC_010226), CPV5 (FJ492743), CPV6 (FJ492744), CPV7 (FJ492742); *Capra hircus* PV (ChPV1) (NC_008032); *Erethizon dorsatum* PV (EdPV1) (AY684126); *Equus caballus* PV (EcPV1) (NC_003748); *Fringilla coelebs* PV (FcPV1) (NC_004068); *Felis domesticus* PV (FdPV1) (NC_004765), FdPV2 (EU796884); hamster oral PV (HaOPV1) (E15111); HPV1 (NC_001356), HPV3 (NC_001588), HPV4 (NC_001457), HPV5 (NC_001531), HPV9 (NC_001596), HPV12 (X74466), HPV13 (NC_001349), HPV14 (X74467), HPV15 (NC_001579), HPV16 (NC_001526), HPV17 (X74469), HPV18 (NC_001357), HPV20 (U31778), HPV21 (U31779), HPV32 (X74475), HPV41 (NC_001354), HPV50 (NC_001691), HPV63 (NC_001458); *Lynx rufus* PV (LrPV1) (AY904722); *Mastomys natalensis* PV (MnPV1) (NC_001605); ovine PV (OvPV1) (NC_001789); *Puma concolour* PV (PcPV1) (AY904723); *Psittacus erithacus* PV (PePV1) (NC_003973); *Procyon lotor* PV (PlPV1) (AY763115); *Panthera leo persica* PV (PlpPV1) (AY904724); *Phocoena spinipinnis* PV (PsPV1) (NC_003348); rabbit oral PV (ROPV) (NC_002232); *Rousettus aegyptiacus* PV (RaPV1) (NC_008298); *Trichechus manatus latirostris* PV (TmlPV1) (NC_006563); *Ursus maritimus* PV (UmPV1) (NC_010739); *Uncia uncia* PV (UuPV1) (DQ180494). Numbers at internal nodes represent the posterior probability support values. Only posterior probabilities above 0.5 are shown.

only around 50 % (46–54 %) identity. Throughout most protein alignments, CPV3, CPV4 and CPV5 revealed higher intra-clade identities than the other two proposed clades of COPV and CPV6 or CPV2 and CPV7. The E2 of CPV4 is less identical to the one of CPV3 and CPV5 than E2 of COPV is to that of CPV6. Likewise, the L1 of CPV4 is less identical to the one of CPV3 and CPV5 than L1 of COPV is to that of CPV6 or than L1 of CPV2 is to that of CPV7. In conclusion, the pairwise amino acid alignments support the classification of the canine PVs into three clades.

Associated clinical conditions

The association of PVs with certain clinical signs is of special interest. The corresponding information from the previously published and the novel cases was assembled and analysed (Table 3). CPV3, CPV4 and CPV5 appear to be similar as they are all associated with pigmented plaques that are of a persistent character. COPV and CPV6 were mainly found to be associated with self-regressing lesions. A certain grouping of viruses in terms of their associated clinical conditions seems to be present.

Table 2. Comparison of the ORFs at the protein level

Values are percentage identities.

	E1						
E2	**COPV**	**CPV2**	**CPV3**	**CPV4**	**CPV5**	**CPV6**	**CPV7**
COPV	–	45	42	41	42	65	42
CPV2	24	–	42	40	40	44	70
CPV3	33	27	–	74	81	44	40
CPV4	33	26	54	–	76	44	39
CPV5	34	26	64	53	–	44	39
CPV6	65	26	36	35	34	–	40
CPV7	30	52	30	28	29	32	–
	E6						
E7	**COPV**	**CPV2**	**CPV3**	**CPV4**	**CPV5**	**CPV6**	**CPV7**
COPV	–	32	25	28	23	43	34
CPV2	31	–	31	34	30	39	54
CPV3	40	22	–	62	67	29	29
CPV4	46	25	78	–	56	33	31
CPV5	45	28	80	80	–	32	28
CPV6	59	35	31	40	41	–	42
CPV7	33	72	20	25	29	35	–
	L1						
L2	**COPV**	**CPV2**	**CPV3**	**CPV4**	**CPV5**	**CPV6**	**CPV7**
COPV	–	49	52	54	53	72	50
CPV2	30	–	46	49	50	48	73
CPV3	34	31	–	71	82	51	46
CPV4	34	31	66	–	70	51	48
CPV5	35	31	78	66	–	52	48
CPV6	62	28	35	33	34	–	48
CPV7	29	64	30	29	30	28	–

DISCUSSION

PVs are known to exist with a relatively large genetic variety in humans, but so far the whole genomes of only four CPVs, which group into three distinct clades, have been published. The latter finding supported the hypothesis that there might be a broad variety of CPVs as well. Here, we compared the genomic sequences, features, phylogenetics and disease-associations of these four CPVs with those of three novel PVs in order to gain a better overall picture of CPVs and their characteristics. We identified not only the typical lambda-PV features, such as a large NCR2 in the case of COPV and CPV6, but also possible characteristics of the two other proposed CPV clades.

The size of the CPV genomes was found to differ among the three proposed CPV clades. Those of the lambda-PVs were, as lambda-PVs are in general, the longest. The sequences of CPV2 and CPV7 were still long but clearly shorter than the lambda-PVs. The shortest and, in terms of length, the least varying genomic sequences, were found among the clade of CPV3. This appears to be a parallel to HPVs of the genera alpha, beta and gamma (Supplementary Fig. S1). Size trends could also be seen in all the coding and non-coding regions of the CPV genomes with the most prominent difference seen in the lambda-PVs, where the NCR2 was longest and E2 and E4 were shortest. It is not clear what kind of biological effects may be associated with these, in several cases significant, size differences of the genomes and ORFs. The differences, nevertheless, seem to be mainly clade specific, in analogy to findings in HPVs. However, some trends recognized in HPVs do not seem to be present in CPVs, namely the association of low GC content and a small E2 ORF with a high risk of developing tumours. As only very few members in each proposed CPV clade have been recognized, these trends remain unclear and need to be reconsidered whenever new viruses grouping into these clades are discovered.

The phylogenetic analysis of the CPVs in comparison with other PVs grouped the seven CPVs into three proposed clades. The positions of the clades could be confirmed, but the posterior probability support of the Bayesian phylogeny of the canine clades remains limited, as not many closely related PVs are currently known. This finding nevertheless further supports the hypothesis that animals, like humans, harbour their own set of PVs, which group into different genera that have different characteristics. This hypothesis

Table 3. CPVs and their corresponding clinical conditions

Virus	Genus*	Clinical signs	Previous reports (selection)
COPV	λ	Oral papillomas, inverted papillomas, invasive SCC, asymptomatic infections	M'Fadyean & Hobday (1898); Sundberg *et al.* (1994); Delius *et al.* (1994); Nicholls & Stanley (1999); Ghim *et al.* (2000); Lange *et al.* (2009)
CPV2	U1	Inverted papillomas, exophytic warts	Goldschmidt *et al.* (2006); Yuan *et al.* (2007)
CPV3	U2	Pigmented plaques, *in situ* SCC, invasive SCC	Tobler *et al.* (2006); Lange *et al.* (2009)
CPV4	U2	Pigmented plaques	Tanabe *et al.* (2000); Narama *et al.* (2005); Tobler *et al.* (2008)
CPV5	U2	Pigmented plaques	None
CPV6	λ	Inverted papillomas	None
CPV7	U1	Exophytic warts, *in situ* SCC	None

*U1, Unnamed putative PV genus 1; U2, unnamed putative PV genus 2.

previously arose from the discovery of several BPVs (Campo *et al.*, 1981; Jarrett *et al.*, 1984; Claus *et al.*, 2008; Hatama *et al.*, 2008; Ogawa *et al.*, 2007; Tomita *et al.*, 2007) and recently from the discovery of some CPVs (Tobler *et al.*, 2006, 2008; Yuan *et al.*, 2007). The clade including CPV3, CPV4 and CPV5 as well as the clade including CPV2 and CPV7 should consequently be regarded as genera even though they might temporarily remain unnamed. It can be anticipated that several CPVs that are yet to be discovered will group into these three proposed genera. The discovery of CPVs grouping into other possibly new clades can also be expected, since fragments of PVs, which may not fit into the present clades, were previously found in canine lesions (Zaugg *et al.*, 2005).

Phylogenetic classification of HPVs only partially correlates with clinical presentation (Howley & Lowy, 2007). Similarly, the putative classification of CPVs (Table 3) does not unanimously link with the clinical consequences of infection. Nevertheless, some features seem to be characteristic for some clades. CPV3, CPV4 and CPV5 were extracted from dogs suffering from persisting pigmented plaques and might be associated with these signs of disease. COPV and CPV6 might be associated with transient endophytic or exophytic proliferation but also with asymptomatic infection (unpublished data).

In conclusion, the genomic analysis of three novel CPVs proposes their classification into three distinct genera. The putative characteristics of these genera may be applied to classify other currently unknown CPVs. Their sequences and biological properties might disclose the true nature of PV infections in dogs.

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Table S1
Characteristic features on nucleotide and amino acid level

A

	COPV	CPV2	CPV3	CPV4	CPV5	CPV6	CPV7
GC content	**42%**	**46%**	**51%**	**53%**	**50%**	**40%**	**46%**

B

Predicted nt-feature	**COPV**	**CPV2**	**CPV3**	**CPV4**	**CPV5**	**CPV6**	**CPV7**
E2 binding site (ACC-N_6-GGT)	**203; 321**	**331; 434; 3747; 5259**	**116; 179; 285; 325; 416; 475; 498; 1274; 4501; 5460**	**57; 124; 314; 404; 463; 487; 4506; 5575**	**107; 170; 317; 406; 465; 490; 1270; 2217; 4518; 4762; 5624; 7088**	**88; 252; 320; 390; 845; 2482; 2616; 6233; 6508**	**156; 173; 327; 356; 431; 2736; 5070**
Dyad symmetry repeats * (TTGTTGTTAACAACAA)	**281**	**393**	**454**	**442**	**444**	**275**	**99; 390; 667; 4532; 7507**
Poly adenylation sites (AATAAA)	**24; 5524; 5637; 7055; 7123**	**3; 72; 310; 5469; 6069; 7621**	**4850; 7800**	**4786; 4790; 7741**	**2499; 7809**	**17; 4036; 4250; 5276; 7136; 8179; 8242**	**59; 4512; 4986; 6692; 7271; 7954**
SP1 binding sites (GGCGGG)	**3824**	**5508**	**462**	**552; 3458; 4889; 5116**	**555**	**3455**	**1861; 5198**
NF1 binding sites (CGGAA)	**911; 825; 1686; 2640; 2771; 3098**	**401; 969; 2392; 3155; 3914; 6775**	**204; 230; 301; 522; 2408; 3408; 3633**	**209; 250; 279; 510; 1640; 2260; 4572; 5840; 6260**	**195; 215; 255; 360; 514; 2407; 2729; 3239; 3629; 4166; 4722; 5712**	**155; 3949; 4583; 6464; 6535**	**398; 3464; 5264; 6626**
Tata signals (TATAAA or TATA(A/T)A(A/T))	**354; 1882; 2155; 6599; 7255; 8133**	**192; 449; 3518; 4547; 4555; 4642**	**174**	**429; 6088**	**2722**	**348; 1897; 2170; 4437; 4484; 4627; 4838; 7450**	**38; 448; 1732; 3203; 3512**

*** Modified in all cases but CPV3 and CPV4**
Underlined starting positions are part of the putative origin of replication

C

Predicted aa-feature	**COPV**	**CPV2**	**CPV3**	**CPV4**	**CPV5**	**CPV6**	**CPV7**
ATP-dependent helicase motifs in E1 † (GPPNTGKS)	**426aa/ 2356nt**	**437aa/ 2494nt**	**456aa/ 2642nt**	**457aa/ 2632nt**	**457aa/ 2641nt**	**431aa/ 2365nt**	**434aa/ 2485nt**
metal-binding motifs in E6 ‡ ($CX_2CX_{29}CX_2C$)	**33aa/ 460nt 106aa/679nt**	**25aa/ 568nt 98aa/ 787nt**	**25aa/ 642nt 98aa/ 861nt**	**25aa/ 632nt 98aa/ 851nt**	**25aa/ 635nt 98aa/ 854nt**	**25aa/ 460nt 98aa/679nt**	**25aa/ 560nt 98aa/ 779nt**
metal-binding motifs in E7 ‡ § ($CX_2CX_{29}CX_2C$)	**52aa/ 948nt**	**50aa/ 1035nt**	**59aa/ 1144nt**	**53aa/ 1131nt**	**54aa/ 1140nt**	**52aa/ 945nt**	**50aa/ 1053nt**
pRb binding domain in E7 (LXCXE)	**22aa/ 858nt**	**-**	**28aa/ 1051nt**	**23aa/ 1041nt**	**23aa/ 1047nt**	**24aa/ 861nt**	**-**

† Modified (GPPDTGKS) in all cases but COPV, CPV4 and CPV6
‡ Modified ($CX_2CX_{28}CX_2C$) in case of COPV and CPV6
§ Modified ($CX_2CX_{30}CX_2C$) in case of CPV2 and CPV7

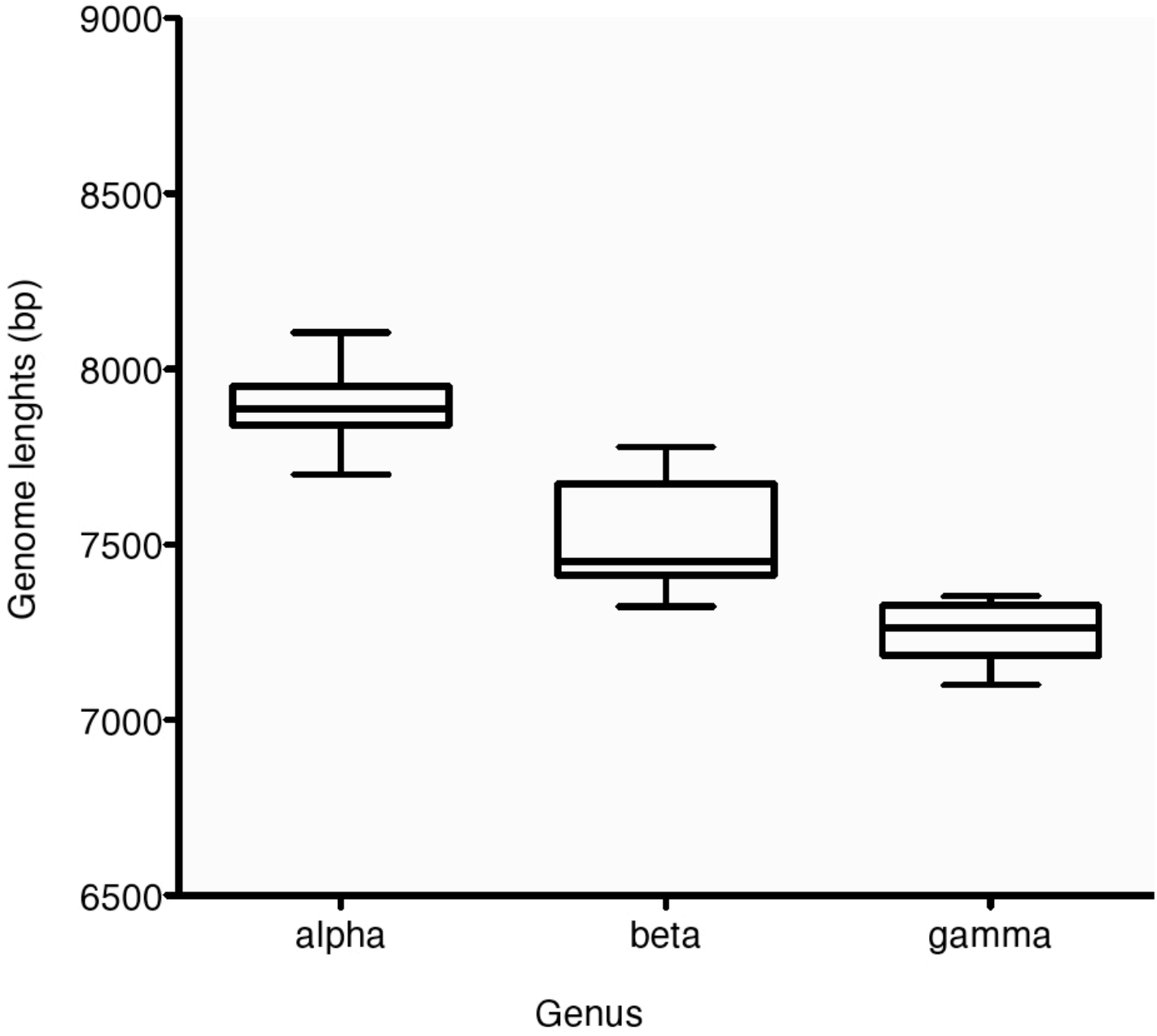

Figure S1: Box plot analysis comparing the distribution of genome sizes among the HPV genera Alpha (HPVs 2, 3, 6, 7, 10, 11, 13, 16, 18, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 39, 40, 42, 43, 44, 45, 52, 53, 54, 55, 56, 57, 58, 59, 61, 62, 66, 67, 68, 69, 70, 71, 72, 73, 74, 77, 81, 82, 83, 84, 90, 94, 97, 102, 106), Beta (HPVs 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49, 75, 76, 80, 92, 93, 96, 98, 99, 100, 104, 105, 107, 109, 110, 111, 113) and Gamma (HPVs 4, 48, 50, 65, 88, 95, 101, 103, 108, 112). The differences among the genera proved significant ($P < 0.05$) in Bonferroni test (PRISM, GraphPad Software).

The clinically healthy skin of dogs is a potential reservoir for canine papillomaviruses

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Own contribution

I was substantially involved in the planning and outlining of this work. I performed part of the molecular biological experiments and a major part of the data analysis. I also did the major part of the writing.

In detail, figures and tables: Table 1 by C. E. Lange, figure S1 by C. E. Lange and K. Tobler, figure S2 by K. Tobler, table S3 by C. E. Lange and K. Tobler

In detail, text: Abstract by C. E. Lange, main text by all authors

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Clinically Healthy Skin of Dogs Is a Potential Reservoir for Canine Papillomaviruses▽#

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Papillomaviruses have been linked to several skin disorders in the dog. In order to have a suitable diagnostic tool for canine papillomavirus detection, eight PCRs with published primer combinations were evaluated. The most sensitive PCR was used to demonstrate that papillomavirus DNA can be detected on nonlesional skin of dogs.

Papillomaviruses (PVs) are predominantly species-specific pathogens of humans and animals that can induce benign as well as malignant neoplasias in the skin and mucous membranes (24). PV DNA has also been detected on the skin of clinically healthy humans and certain animal species (1–4, 6, 7, 16, 17). Likewise, clinically healthy dogs have been shown to carry antibodies against PVs (14). Nevertheless, previous attempts to detect PV DNA on the skin of healthy dogs have been unsuccessful (3).

To address this issue, eight PCR assays with previously published primer combinations targeting either the L1 or the E1 open reading frame (ORF) were assessed (Table 1 and see references therein). These PCR assays were evaluated for their ability to detect the DNA of the seven classified canine PVs (CPVs) (5, 9, 12, 20–22). Furthermore, the sensitivity and specificity of each assay were determined in the same context.

Eight different published, primarily broad-range primer pairs were tested (Table 1). Three of them target conserved regions in the L1 open reading frame, namely, canPVf/FAP64, FAP59/FAP64, and AR-L1F1/AR-L1R3, while five of the primer pairs target conserved stretches in the E1 ORF, namely, CP4/CP5, PPF1/CP5, PapF/PapR, AR-E1F1/AR-E1R2, and AR-E1F2/AR-E1R9. Primers amplifying 585 bp of canine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed to test for host DNA (dogGAPDHf, GGT GAT GCT GGT GCT GAG TA; dogGAPDHr, GAC CAC CTG GTC CTC AGT GT). RedTaq (Sigma, Buchs, Switzerland) ready reaction mix was used according to the manufacturer's recommendations. Three different protocols were used. In the cases of the primer combinations canPVf/FAP64, AR-L1F1/AR-L1R3, AR-E1F1/AR-E1R2, and AR-E1F2/AR-E1R9, 10 min of initial denaturation at 94°C was followed by 45 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. The protocol concluded with a final elongation step of 72°C for 10 min. In the case of FAP59/FAP64 and dogGAPDHf/dogGAPDHr, the protocol used started with 3 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C. The protocol used for the primer combinations CP4/CP5, PPF1/CP5, and PapF/PapR also started with 3 min at 94°C but was then followed by 40 cycles of 30 s at 94°C, 30 s at 42°C, and 30 s at 72°C.

To visualize PCR results, 1% agarose gels containing ethidium bromide were used. Images were taken after a run of 35 min in an electric field of 5 V cm^{-1} in Tris-acetate-EDTA (TAE) buffer.

Rolling circle amplification (RCA) was used to test for circular, potentially papillomaviral DNA (18). DNA (1 μl) was used for RCA in a TempliPhi amplification kit (General Electrics Biosciences, Glattbrugg, Switzerland). The protocol supplied by the manufacturer was used, with slight modifications. Namely, 1 μl of 10 mM deoxynucleoside triphosphates (dNTPs) was added, and the reaction time was prolonged to 16 h at 30°C. Two templates were used alternatively for the evaluation: one complete genomic clone of CPV1 in a pBluescript II KS+ vector (Stratagene, La Jolla, CA) and one pET-DEST42 vector (Invitrogen, Basel, Switzerland) containing the entire L1 coding sequence of CPV1. The amplified DNA was digested with the restriction endonuclease EcoRI or EcoRV, respectively. To visualize results, 1% agarose gels containing ethidium bromide were used. Images were taken after 90 min in an electric field of 5 V cm^{-1} in TAE buffer.

To evaluate the spectrum of the primers, clones or PCR products of the target regions from the seven PVs were used as templates. Whole genomes cloned into pBluescript II KS+ (Stratagene, La Jolla, CA) were used in the cases of CPV1 (EcoRI), CPV3 (SacI), CPV5 (ClaI), CPV6 (EagI), and CPV7 (HinDIII); a partial genomic clone was used in the case of CPV4 (KpnI). PCR products of E1 or L1 target regions were used in the cases of CPV2 and CPV4 (E1 CPV2 forward, GTG GTT TGT TGT GCA TGA GG; E1 CPV2 reverse, CCA AAG TCC ATG GTT CAT CC; L1 CPV2 forward, TGA TAC ACA GGA AGC GCA AA; L1 CPV2 reverse, TGC CTT CCT TCT TTT CTT TGA; E1 CPV4 forward, ACC CAG GAG AGG GTA CCA GT; E1 CPV4 reverse, CCC TCG TCC TCT TGA TCA CT). For the evaluation of the dogGAPDH primers, a PCR product of the whole dogGAPDH (forward, ATG GTG AAG GTC GGA GTC AA; reverse, TTA CTC CTT GGA

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TABLE 1. Primers and detection levels indicating the minimum concentration of molecules required for detection of CPVs

Target gene	Primer names and sequences[a]	No. of molecules/reaction required to detect[b]:						
		CPV1	CPV2	CPV3	CPV4	CPV5	CPV6	CPV7
L1	canPVf/FAP64 (13); CTTCCTGAWCCTAAYMAKTTTGC/CCWATATCWVHCATNTCNCCATC	10^1	10^4	10^1	10^1	10^2	10^1	10^4
	FAP59/FAP64 (10); TAACWGTNGGNCAYCCWTATT/CCWATATCWVHCATNTCNCCATC	10^4	10^6	10^9	10^8	10^8	ND	10^8
	AR-L1F1/AR-L1R3 (19); TTDCAGATGGCNGTNTGGCT/CATRTCHCCATCYTCWAT	10^4	10^6	10^5	10^6	10^6	10^5	10^6
E1	CP4/CP5 (11); ATGGTACARTGGGCATWTGA/GAGGYTGCAACCAAAAMTGRCT	10^1	10^4	10^5	10^1	10^2	10^1	10^3
	PPF1/CP5 (11); AACAATGTGTAGACATTATAAACGAGC/GAGGYTGCAACCAAAAMTGRCT	10^4	10^8	ND	10^7	ND	10^4	ND
	PapF/PapR (23); ATGGCGGATAAAAAAGGTA/AACAGCTGTTTTTTAGCTTTTTT	10^5	ND	ND	ND	ND	10^6	ND
	AR-E1F1/AR-E1R2 (19); CAGGGVMWTTCCCTGBARYTGTTYC/TCATANGCCCACTGNACCAT	10^1	10^5	10^6	10^7	10^6	10^4	10^7
	AR-E1F2/AR-E1R9 (15); ATGGTNCAGTGGGCNTATGA/CATTWGTDGTDAYMAGSAKRGGVGGGCA	10^1	10^3	10^2	10^7	10^6	10^3	10^6

[a] Nomenclature for nucleotide symbols is according to Cornish-Bowden (8).
[b] ND, not detected.

GGC CAT GTA) served as the template. To determine the approximate detection levels of the PCRs, serial dilutions were made from 10^9 down to 10^1 molecules of every template. For that purpose, DNA concentrations of the template stock solutions were measured using a spectrophotometer (ND-1000; Thermo Scientific, Zurich, Switzerland). According to the size of the individual templates, the number of molecules per microliter was calculated. DNA from a keratinocyte cell culture (Bex), being free of any known PV DNA, served as a negative control for PV primers (see Fig. S1 in the supplemental material). Thus, DNA was extracted from 10^6 cells with a DNeasy extraction kit (Qiagen, Hombrechtikon, Switzerland), resulting in 83 ng/μl. Eight serial dilutions (1:10) were made.

The test population of 95 dogs was recruited from patients at the small animal hospital of the University of Zurich displaying no clinical signs of any described kind of papillomatosis. To obtain skin samples from these dogs, fresh cytobrush sticks were used in the oral cavity and on the interdigital skin. The cytobrushes were rubbed six turns on the target regions and afterward placed in a 1.5-ml Eppendorf tube containing 1 ml of sterile 0.9% NaCl. The samples were kept at 4°C for no longer than 24 h and were then stored below −18°C until extraction. Before DNA extraction, each cytobrush was flushed repeatedly with the 0.9% NaCl it had been kept in. To concentrate all cells and cell debris at the bottom of the tube, tubes (still containing the cytobrush tip) were centrifuged at 15,000 × *g* for 10 min. The cytobrush tip and the 0.9% NaCl were then carefully removed, except for about the last 25 μl. DNA was afterward extracted using the DNeasy extraction kit (Qiagen, Hombrechtikon, Switzerland) by following the manufacturer's instructions, finally eluting DNA in 100 μl sterile water. Extraction and handling of this sample DNA were performed entirely separately from any other PV research.

All sampling was carried out in accordance with the Swiss regulations about research on animals.

As shown in Table 1, PCR with the L1 primer combination canPVf/FAP64 detected five of the seven CPVs at a target concentration of 10^2 molecules/reaction or less, while CPV2 and CPV7 DNA was still detected at a target concentration of 10^4 molecules/reaction. Assays with E1 primers CP4/CP5 performed similarly but needed more target to detect CPV3. All tested PCRs with other primer combinations showed less sensitivity and/or less specificity (Table 1; see also Fig. S1 in the supplemental material). The reason may be that some primers aligned well with certain CPV sequences but poorly with others (see Fig. S2 in the supplemental material). RCA is a frequently used alternative method to amplify PV DNA (18). Evaluation of its sensitivity revealed that it required a minimum of 10^6 molecules to successfully amplify PV DNA (data not shown); thus, RCA was not favored for the detection of CPV DNA on healthy skin.

Based on the primer evaluation, the canPVf/FAP64 PCR was chosen to assess cytobrush samples from the skin and oral mucosa of 95 asymptomatic dogs. A dogGAPDH PCR assay (requiring 10^4 molecules for positive detection) was used as an amplification control. This PCR provided a positive signal with all samples taken from the oral cavity and with 81% of samples taken from the interdigital skin (see Table S3 in the supplemental material). CPV DNA was detected in more than 50% of the dogs (see Table S3 in the supplemental material), i.e., in 23% of oral samples whose complementary skin samples were negative, 14% of skin samples (oral samples negative), and 15% of samples from both locations. In 48% of the dogs, PV DNA was detected neither in skin nor in oral samples. Interestingly, in two cases, putative PV DNA was amplified from the skin with canPVf/FAP64 although the dogGAPDH PCR assay had been negative. A number of randomly selected products arising from canPVf/FAP64-mediated PCR were sequenced. The sequences obtained from two oral and three skin samples were clearly identified as CPV1. In contrast, the amplification products of five oral and two skin samples did not yield meaningful sequencing results, most probably due to the presence of DNA from more than one PV type in the sample.

In conclusion, the sensitivity and specificity for each of eight

previously described PV primer pairs was assessed in the context of PCR for the detection of CPV DNA. Application of a broad-range, highly sensitive primer pair suggests that the clinically unaffected skin and oral cavity of dogs might be a reservoir for canine papillomaviruses.

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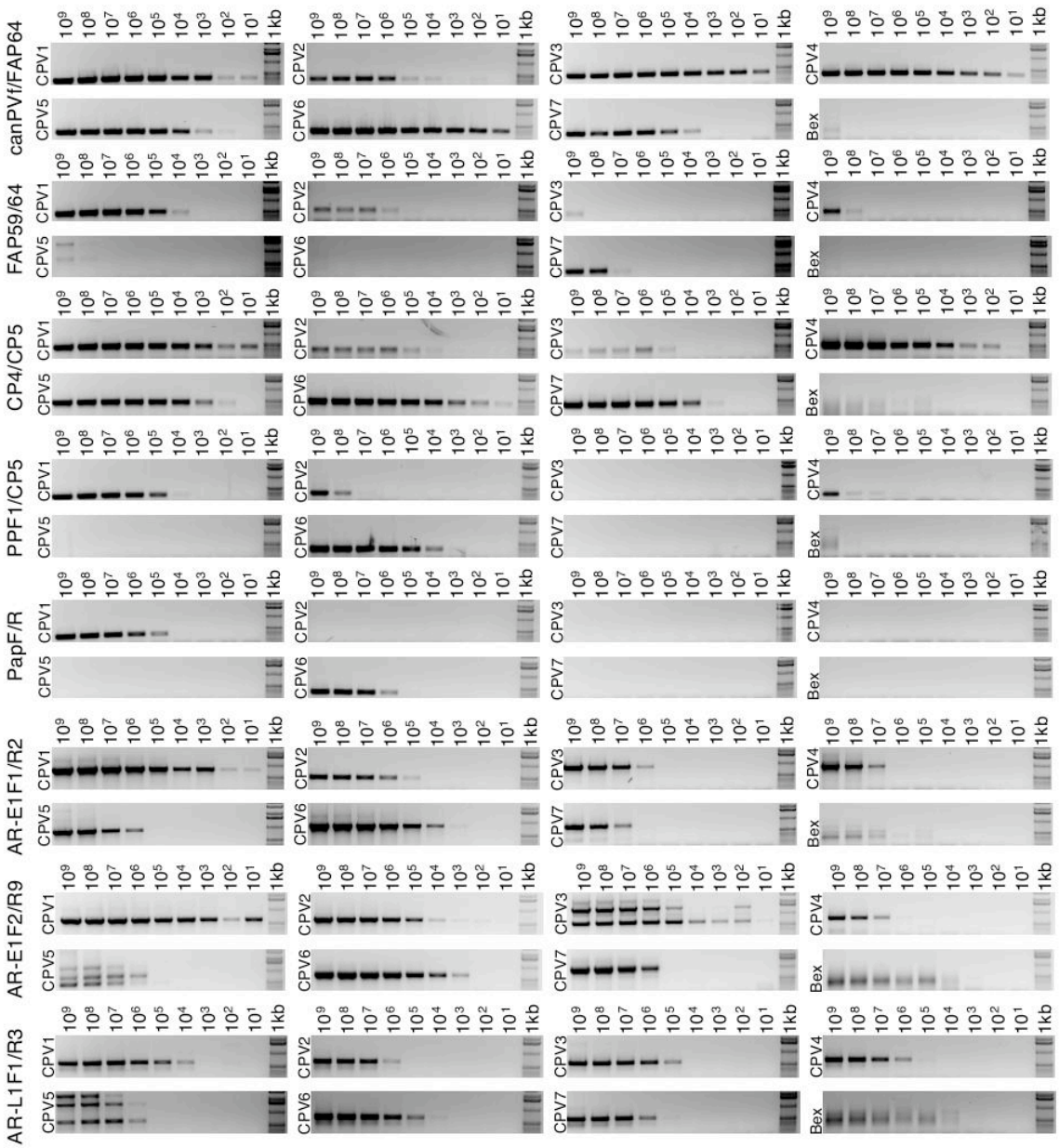

Figure S1: Evaluation of PV primers. PCR with eight primer combinations tested on template DNA of seven CPVs and a canine keratinocyte cell culture (Bex). CPV template indicated as molecules per sample, in case of Bex 1:10 serial dilutions of a 10^6 cell equivalent.

FAP59	TAACWGTNGGNCAYCCWTATT
CPV1 (6964-6985)	.t.....t..
CPV2 (6332-6353)	.c.....a.....
CPV3 (5884-5905)	.g.....a.....t..
CPV4 (5922-5943)	.g..c.....a.....t..
CPV5 (6294-6315)	.g.....a.....t..
CPV6 (6495-6516)	.....ctg.
CPV7 (6162-6183)	.....a.....

FAP64	CCWATATCWVHCATNTCNCCATC
CPV1 (7434-7457)	.....
CPV2 (6790-6813)	....c.....g.....
CPV3 (6348-6371)	.....c..c.....
CPV4 (6377-6400)	.....
CPV5 (6752-6775)	.....c....g.....
CPV6 (6968-6991)	.....
CPV7 (6620-6643)	....c.....g.....

canPVf	CTTCCTGAWCCTAAYMAKTTTGC
CPV1 (7068-7091)	.....a..c.....
CPV2 (6427-6450)	..c..c.....a.....
CPV3 (5985-6008)	..c.....
CPV4 (6023-6046)	.....c.....gc.....
CPV5 (6395-6418)	..c.....c..a.....a.....
CPV6 (6599-6622)	t.g.....c.....
CPV7 (6257-6280)	t.....c..a.....

CP4	ATGGTACARTGGGCATWTGA
CPV1 (1737-1757)	.....t.....t.....
CPV2 (1749-1769)	.....g.....t.....
CPV3 (1746-1766)	.....c.....g.....
CPV4 (1830-1850)	.....g.....c.....
CPV5 (2149-2169)	.....g.....
CPV6 (1645-1665)	.....t.....
CPV7 (1722-1742)	.....t.....t.....

CP5	GAGGYTGCAACCAAAAMTGRCT
CPV1 (2178-2200)	.....g.....
CPV2 (2190-2212)	.g..gct..g.....g.....
CPV3 (2184-2206)	.t.....g...g.....
CPV4 (2268-2290)	.t...att.g...g.....
CPV5 (2587-2609)	at.....g.....t.....
CPV6 (2083-2105)	a.....t.....
CPV7 (2160-2182)	....actt.g.....

PPF1	AACAATGTGTAGACATTATAAACGAGC
CPV1 (1877-1904)	.....c...t.....aa...
CPV2 (1889-1916)	..a....gtc..g.....c...aa.ca
CPV3 (1886-1913)	.gtg...gtc.ag.....c.gg..t..
CPV4 (1970-1997)	...c...gtc.....c.....ga....
CPV5 (2289-2316)	tgtt...gt..ag.....ga.g..
CPV6 (1785-1812)	.....c...t.....a....
CPV7 (1862-1889)	..a....gtc.ag.....c...aa.ca

PapF	ATGGCGGATAAAA------AAGGTA
CPV1 (816-835)	.....c..g.. .....
CPV2 (795-820)	.....c..agg.ggtgaaag.....
CPV3 (732-757)	....a..g.g.c.tagattc.....
CPV4 (813-838)	....a..g.g.cctgggatc.....
CPV5 (1132-1157)	....ac.g.g..ctagattc.....
CPV6 (706-725)	....
CPV7 (774-799)	.....a..ag..ggtgaaag.....

PapR	AACAGCTGTTTTTTAGCTTTTTT
CPV1 (1128-1151)	.....c.....a.c.....
CPV2 (1137-1160)	..a..tc..cgc..gctg.g...
CPV3 (1054-1076)	c.a.ag.c.gcgc.ttgcac.c.
CPV4 (1132-1154)	c.acaggcg.c.c.ttgc.g...
CPV5 (1451-1473)	c.ataacc.gcgc.ttgc.g...
CPV6 (1015-1038)	.....c.....t..c.....
CPV7 (1098-1121)	..t..ac.ccgc..gctg.....

AR-E1F1	CAGGGVMWTTCCCTGBARYTGTTYC
CPV1 (969-994)	.....
CPV2 (978-1003)	..t.a.ggacatgca.gtg....aa
CPV3 (900-925)	.....aa.ct..a..g.
CPV4 (978-1003)	.....g.....a..g.
CPV5 (1297-1322)	.....aa.ct..a..g.
CPV6 (856-881)	.....a....
CPV7 (939-964)	..t.a.g.gcatgca.g...a..aa

AR-E1R2	TCATANGCCCACTGNACCAT
CPV1 (1737-1757)	.....
CPV2 (1749-1769)	.....
CPV3 (1746-1766)	.....
CPV4 (1830-1850)	.....
CPV5 (2149-2169)	.....
CPV6 (1645-1665)	.....t.....
CPV7 (1722-1742)	.....t.....

AR-E1F2	ATGGTNCAGTGGGCNTATGA
CPV1 (1737-1757)	.....
CPV2 (1749-1769)	.....
CPV3 (1746-1766)	.....
CPV4 (1830-1850)	.....
CPV5 (2149-2169)	.....
CPV6 (1645-1665)	.....a.....
CPV7 (1722-1742)	.....a.....

AR-E1R9	CATTWGTDGTDAYMAGSAKRGGVGGGCA
CPV1 (2337-2365)	.....
CPV2 (2349-2377)	.....a.....ca.
CPV3 (2343-2371)	t....c.....g.....t.....a..
CPV4 (2427-2455)	t...gc....c....at.at.....a..
CPV5 (2746-2774)	tg.....at.....a..
CPV6 (2242-2270)	.....t.....t..a..
CPV7 (2319-2347)	tg....a.....t..tag

AR-L1F1	TTDCAGATGGCNGTNTGGCT
CPV1 (6830-6851)	.....
CPV2 (6195-6216)	.....t.....a.
CPV3 (5750-5771)	.....g.
CPV4 (5788-5809)	.....g.
CPV5 (6160-6181)	.....g.
CPV6 (6361-6382)	.....
CPV7 (6025-6046)	.....t.....a.

AR-L1R3	CATRTCHCCATCYTCWAT
CPV1 (7428-7446)	.....
CPV2 (6784-6802)	...c.g.....
CPV3 (6342-6360)	.....g...
CPV4 (6371-6389)	.....g...
CPV5 (6746-6764)	.....g...
CPV6 (6962-6980)	.....g...
CPV7 (6614-6632)	...c.g.....

Figure S2: Multiple sequence alignments of primers and their binding sites. Oligo sequences are written from 5' to 3' in upper case letters according the IUPAC code. Genomic positions of the binding sites are put in brackets. Positions matching to the oligo sequences are shown as dots, positions not matching to the oligo sequences are shown as lower case letters.

S3: PCR screening for PV DNA with canPVf/FAP64 primers on healthy skin. dogGAPDH PCR was included as internal control. Numbers of individual dogs are divided into the sixteen combinations of positive and negative PCR results for GAPDH and PV DNA of oral and interdigital samples.

	PV DNA oral pos / paw pos	PV DNA oral pos / paw neg	PV DNA oral neg / paw pos	PV DNA oral neg / paw neg
GAPDH oral pos / paw pos	14	14	10	39
GAPDH oral pos / paw neg	0	9	2	7
GAPDH oral neg / paw pos	0	0	0	0
GAPDH oral neg / paw neg	0	0	0	0

Generation of canine keratinocyte cell lines harbouring canine papillomavirus DNA over more than 100 passages

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In preparation

Own contribution

I was involved in the planning and outlining of this work. I performed the major part of the molecular biological experiments as was as of the data analysis. I also did the major part of the writing.

In detail, figures and tables: Figure 1 by C. Lange, figure 2 by Marco Franchini, Figure 3 by C. E. Lange and E. Vetsch, Table 1 by C. E. Lange

In detail, text: Manuscript by C. E. Lange

Generation of canine keratinocyte cell lines harbouring canine papillomavius DNA over more than 100 passages

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Abstract

Papillomaviruses are small DNA viruses that can be linked to asymptomatic infections, but also to various benign and several malignant disorders of the skin and mucous membranes. Many such viruses and associated diseases have been described in humans, but little is known about the similar conditions in animals such as the dog. Consequently our aim was to establish a cell culture model, in order to have a tool for future studies on the pathogenesis of papillomavirus induced conditions. For this purpose DNA of the canine papillomaviruses CPV1, CPV3 and CPV5 was introduced into canine keratinocytes using the Herpesvirus Amplicon system. Successfully transduced cells were enriched based on GFP expression using fluorescence activated cell sorting. The cells were subsequently monitored for GFP expression, for the presence of viral DNA and for DNA transcription. Cells could be found positive for GFP, DNA and RNA over more than one hundred passages post transduction, indicating that the cell lines stably harbour papillomavirus DNA. In conclusion keratinocyte cell lines containing and transcribing the DNA of canine Papillomaviruses were generated. These cell lines may serve as a model for future studies on pathogenic mechanisms of papillomaviruses.

Introduction

Papillomaviruses (PVs) are DNA viruses with a double stranded circular genome of about 8kbp in size contained in a nonenveloped icosahaedral capsid of about 50nm. PVs primarily infect epithelial cells and can cause subclinical infections but also benign proliferations which may progress into malignant tumours (Chen et al., 1982; Baker et al., 1991; Jablonska et al., 1997; zur Hausen, 1999). The most intensively studied PV associated disease is the cervix carcinoma, which can be induced by several PV types, most frequently by the human PVs (HPVs) 16 and 18. These two viruses and their effects on the organism have consequently been studied in great detail, as the cervix carcinoma is one of the most abundant cancers in women worldwide (zur Hausen, 1999). Not all of the currently more than 150 known HPV types have been intensely studied as PVs can not easily be propagated in cell culture and less is for example known about the PVs of the Beta genus. PVs belonging to this genus are involved in various diseases of the skin, which may even be more common than cervix tumours. However PV associated skin diseases rarely progress to malignancy and mechanisms in these cases are only partially understood (Sterling, 2005).

PVs and associated diseases have also been identified in animals, and it can be assumed that each species harbours its own set of PVs as they are predominantly species-specific pathogens.

PV associated lesions in dogs have been described in the past (Nagata 1995, Nicholls and Stanley, 1999). Although the association of distinct PVs with each of the observed conditions was suspected years ago, support for this hypothesis was found only recently (Zaugg et al., 2005; Tobler et al., 2006; Yuan et al., 2007,). To date entire genomic sequences of seven canine papillomaviruses (CPVs) have been determined (Lange et al., 2009). These CPVs were grouped into the three genera Lambda, Tau and Chi based on the nucleotide sequences of their L1 open reading frame (ORF) (Bernard et al., 2010). The associated lesions range from transient warts over persistent benign pigmented or unpigmented tumours, to in situ and invasive squamous cell carcinomas (SCCs).

Although the course of infection has repeatedly been observed and described in case of CPV1 (Nicholls and Stanley, 1999), and various data support the role of CPV2 in the development of tumours (Yuan et al., 2006; Goldschmidt et al., 2006, Lange et al., 2010), little is known about the underlying mechanisms in any CPV associated pathology.

To address the question what effects CPV DNA has on their target cells, a model could be of great value. To generate such a model for CPV infection we choose a canine keratinocyte cell line (Kolly C. 2005). DNA of three CPVs was selected to be introduced in the cells, one from the Lambda genus (CPV1) and two from the Chi genus (CPV3, CPV5). The Herpes Amplicon system was used to transduce keratinocytes and GFP signal served as a tool for sorting and assessment of transduction (Fraefel et al., 1996).

Materials and Methods

Cloning

For the purpose of rolling circle amplification (RCA) (Rector et al., 2004), a TempliPhi Amplification kit (General Electrics Biosciences) was used. The protocol supplied by the manufacturer was used, with slight modifications: 1 µl of dNTPs (10 mM) was added and the reaction time was prolonged to 16 h at 30°C. One microliter of DNA extract served as template. In order to amplify the PV genomes of CPV1, CPV3 and CPV5 without their L1 and L2 ORFs according primers adding a SbfI restriction site were designed (Table 1). A Phusion (Finnzymes) polymerase (0.5µl) was used adding 0.5µl of each primer (10µM), 5µl buffer HF (Finnzymes), 0.5µl dNTPs (10mM), 3.75µl Betaine (5M), 1.25µl DMSO and 12µl sterile water to 1µl of template (RCA product 1:100). Conditions for the PCRs were chosen as follows: 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 4 min. A final elongation step, 10 min. at 72°C concluded the program. PCR products were subcloned into HSV-1 amplicon plasmids containing a GFP gene under a 4/5 promoter and an ampicillin resistance gene (D'Antuono et al., 2010). A new restriction site was introduced into the HSV-1 amplicon plasmid using a palindromic oligonucleotide that destroys the NdeI site and introduces a SbfI site (Table 1). Thus the PV DNA remains under its own and GFP independent under the 4/5 promoter. E. coli DH10B cells were transformed to multiply the construct, growing them in LB medium containing 100µM ampicillin. DNA was harvested using a DNA maxi kit (Qiagen). Helper virus-free stocks of the vectors were prepared (Fraefel et al., 1996; Saeki, et al., 1998; Saeki et al., 2001). The HSV-1 genome was provided in *trans* by a bacterial artificial chromosome (BAC) containing the HSV-1 genome with deletions in the DNA cleavage/packaging signals and the essential ICP27 gene (fHSVpacICP27) (D'Antuono et al., 2010).

Cell culture

To observe the effects of CPVs on their primary target cells a canine keratinocyte cell culture deriving from a Beagle was chosen (Kolly C. 2005). The cells were cultured in CnT-09 medium (Cell`n`Tec) in 25cm^3 cell culture flasks (TPP). Cells were split 1:20 every 3-4 days. Transduced cells were split 1:5 or 1:10 over the first 33 passages, afterwards 1:10 continuously.

For RAFT culture growth Milicell 12mm wells (Millipore) were inserted into a 24 well plate. 10^6 cells were seeded per well and cultured in CnT-09 medium for three days before cells were exposed to air (5% CO_2). Medium was subsequently supplemented exclusively from underneath and changed twice a week. RAFT cultures were harvested two weeks after being emerged, formalin fixed and paraffin embedded for further processing.

Transduction

To introduce the PV specific DNA into the canine keratinocytes 10^5 cells were seeded per well of a 24 well plate (TPP) in CnT-09 medium one day pre transduction. A volume of 200µl of unconcentrated Amplicon preparation with titers of around 5 x10^5 TU/ml was applied on the cells for 90 minutes and washed away afterwards.

FACS

The transduced cells were sorted for GFP using a FACSAria II (BD) machine. First sorting took place twelve days post transduction (enrichment), successive sortings 23 and 53 days post transduction (purity). Gating was orientated on untransduced keratinocytes.

PCR (DNA/RNA)

To extract DNA from the cultures 10^6 cells of each cell line were taken and processed using the QIAamp DNA extraction kit (QIAGEN) according to the manufacturer's instructions for cultured cells. For RNA extraction a kit (Machery & Nagel) was used according to the manufacturer's instructions. Additionally the RNA extract was incubated with DNase I (Roche) for 30 minutes. For reverse transcription of RNA a kit (Promega) was used according to the manufacturers' instructions.

Primers were designed to amplify DNA of the main early ORFs of CPV1, CPV3 and CPV5, namely E6, E7, E1 and E2 by PCR (Table 1). RedTaq polymerase ready reaction mix (Sigma) was used according to the manufacturer's recommendations.

PanAAV primers were constructed based on the sequences of seven human AAVs (NC_002077, NC_001401, NC_001729, NC_001829, NC_006152, NC_006260, NC_006261) as well as the AAV of the cow (NC_005889), two avian AAVs (NC_006263, AY629582) and the goose parvovirus (NC_001701) (Table 1). RedTaq polymerase ready reaction mix (Sigma) was used according to the manufacturer's recommendations. Conditions for the PCRs were chosen as follows: 94°C for 5 min followed by 39 cycles of 94°C for 30 sec, 60°C for 1 min and 72°C for 30 sec. Wildtype human AAV2 DNA served as positive control.

Results

In order to establish a model system for CPV infection, genomic DNA lacking the structural genes of CPV1, CPV3 and CPV5 was amplified and cloned into herpesvirus amplicon plasmids. The plasmids were packaged into herpesvirus particles and used to transduce canine keratinocytes (Kolly C. 2005). To rule out that the cell line used in the experiment contains any PV DNA, PCR and RCA were applied to amplify potential PV DNA. No evidence of PV DNA was found prior to transduction. Additionally, the cell line was tested for the presence of DNA of a putative Adeno associated virus; no such DNA was amplified from the cell line (data not shown).

To determine the completeness of the constructs used, the CPV inserts were sequenced. The CPV1 insert was found to differ in from the published sequence in two positions located in the second non coding region, namely nucleotides 4617 and 4626. In case of CPV3 nucleotide 680 of the E1 ORF turned out to be an adenine instead of a guanine, thus substituting the non-conserved E1 amino acid 227 Serin by Asparagin. The CPV5 insert was found to be identical with the published sequence.

Four days post transduction a GFP signal could be observed in 10-15% of the cells under the UV microscope. After three FACS steps the ratio of GFP positive cells was above 70% in each of the cell lines transduced with constructs containing viral DNA (Figure 1). In the cells transduced with the control amplicon no GFP signal was present after the first sorting, consequently no further sorting was conducted. The amount of cells being GFP positive upon microscopic observation decreased gradually over the following passages as well as the intensity of the GFP signal. After 68 passages still several CPV3 transduced cells expressing GFP could be identified, more than 10% in case of the CPV1 transduced cells and even more than 50% in case of CPV5 transduced cells. However, upon FACS assessment after 105

passages more than 90% of CPV5, more than 80% of CPV1 and about 1% of CPV3 transduced cell lines were found to be GFP positive (Figure 2).

To verify that the transduced cell lines contain the DNA of the main ORFs E6, E7, E1 and E2 and of the GFP gene according primers were constructed and PCR was performed (Table 1). DNA of all these genes was amplified in case of the CPV1, CPV3 and CPV5 transduced cell lines (Figure 3). The same primers were used for RT PCR to demonstrate transcription of viral genes. In case of all three viral constructs cDNA of the E6, E7, E1 and E2 ORF was amplified after DNAase treatment and reverse transcription. No DNA was amplified in the controls without reverse transcriptase (Figure 3). The cultures were repeatedly tested for DNA and RNA up to 105 passages post transduction and were consistently positive in the PCR.
The presence of the entire inserts within the cell lines was confirmed after 105 passages using overlapping PCRs in case of all three CPV containing cell lines.

To test whether the presence of the introduced viral DNA has any effects on the growth and differentiation of the keratinocytes, untransduced and transduced cell lines were grown in RAFT culture 43 passages post transduction. No morphological differences between transduced and untransduced cell lines were detectable (data not shown). The application of specific markers for differentiation, multiplication and apoptosis namely Lamin A, Loricrin, Ki-67 and P53 did not reveal statistically significant differences in the amount of marker-tagged cells. No cells positive for GFP signal or GFP immunostaining were seen in any preparation.

Discussion

While CPVs have been shown to be associated with several skin disorders little is known about the underlying mechanisms. Consequently the aim of this project was to generate a tool that could help revealing effects of PV DNA on their natural host cells.
Three viruses were chosen for this study: CPV1 as it is the best studied and possibly most abundant CPV, CPV3 as a causal relation with cancer has been suggested and CPV5 as it is the closes known relative to CPV3. As PVs cannot simply be propagated in cell culture many strategies to study their effects on cells have been applied (Meyers and Laimins, 1994; Conway and Meyers, 2009; Liebertz et al., 2010). We considered and tested several transfection strategies, including chemical transfection with several different agents and electroporation. The transfection rates were at the best around 10-20% (data not shown).

However, as the survival rate of GFP positive cells after sorting was very poor we decided to use the Amplicon system as this is a very gentle method of introducing DNA of interest into target cells. The system also uses GFP as marker gene for successfully transduced cells. This enables relatively easy tracking and also sorting for GFP expressing cells. It is unlikely that the empty vector would replicate alone, because although it does contain an HSV origin of replication (ORI) it lacks the gene for the according HSV DNA polymerase. This was most probably the reason for the loss of GFP signal in the cells transduced with the empty plasmid. The plasmids containing the PV DNA on the contrary carry the PV regulatory region, which harbours the PV ORI. As PVs utilize the host DNA polymerase in association with the geneproducts of E1 and E2 all necessary machinery should be available in the accordingly transduced cells. It remains unclear, in which form the heterologous DNA exists in the cells. It was introduced in linear, concatemeric form, but might have formed circular plasmid DNA or could eventually have integrated into the host genome. The according experiments where however inconclusive. No circular DNA could be amplified via RCA, what might be due to a copy number below the detection level of RCA (Lange et al., 2011). The completeness of the insert was confirmed by PCR. Consequently homogeneous cell lines with integrated PV genomes are unlikely, as integration is usually associated with the destruction of a PV ORF, like E2 in case of HPV18 (zur Hausen, 2002; Howley and Lowy, 2007).

In the cells transduced with the plasmids containing the DNA of CPV1, CPV3 and CPV5 the GFP signal could be used for sorting, resulting in cell lines with a high proportion of GFP positive cells. Although the signal intensity decreased over time, the cell lines harbouring CPV1 and CPV5 were GFP positive in FACS to a high proportion even after more than 100 passages. However, even with restrictive sorting the proportion of fluorescent cells never reached 100%. This might be due to cells, that were sorted incorrectly, cells that may have lost the DNA during cell division or possibly due to the inability to express GFP at certain stages.

As it was observed that the cell lines expressed GFP up to 105 passages post transduction, it can be concluded, that these cells stably harbour the DNA.

As GFP has been merely a tool for FACS and for optical assessment, the main focus is the PV DNA that was brought into the keratinocytes. To check for PV DNA specific primers for the four main ORFs had been made for each of the three viruses. DNA was extracted from cells at different time points and PCR was continuously positive up to 105 passages post transduction. DNA of GFP and canine p53 coding sequences was also amplified from these cells. This supports the assumption, that the cell lines expressing GFP also harbour the DNA of CPV1,

CPV3 and CPV5 respectively. The transcription was also assed in parallel based on the same genes and primers. As RNA could be detected as well, and as the DNA is successfully contained in the cell lines over many passages, it can be concluded, that the viral genes are active in the three cell lines.

As the papillomavirus lifecycle is closely linked to the lifecycle of keratinocytes, the replication and gene expression of PVs depends on the stage of the infected cells (Oriel, 1971; zur Hausen, 1996). As monolayers may only represent the basal layer of keratinocytes a three dimensional model with the different stages of the keratinocyte lifecycle might trigger viral programs that are not active in continuously replicating cells. We applied the RAFT culture model for this purpose and used some markers for cellular processes. As no differences could be seen in comparison to untransduced cells, several possible explanations may exist. As the development of papillomaviral disorders often seems to be a process with a long asymptomatic phase, the experiment might have been performed in a phase of infection that is too early. It is also possible, that the time the cells where allowed growing as a RAFT culture was to short. Besides these it might also be considered, that the virus DNA alone is not sufficient to induce changes in keratinocytes. More experiments in these directions are therefore warranted.

In conclusion, we can resume, that we successfully transduced keratinocyte cell lines with the DNA of CPV1, CPV3 and CPV5 respectively. The DNA of the three viruses is stably contained in the cell lines over more than 100 passages and the main genes age transcribed. Thus we have generated cell lines that can be very useful in addressing questions about the effects of CPV DNA on their natural target cells, to improve the understanding about PV pathology in general and in dogs in particular.

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Figure legends

Figure 1

Canine keratinocytes 7 passages after transduction. Cell lines transduced with CPV1 (A, B), CPV3 (C, D) and CPV5 (E, F) in translight and under UV for GFP detection. 10x Objective of a Zeiss Axiovert S1000 microscope.

Figure 2

FACS assessment of canine keratinocytes. Non transduced keratinocytes served as control to set gating parameters. CPV1, CPV3 and CPV5 transduced keratinocytes 105 passages after transduction. FL1-H indicates GFP.

Figure 3

PCR on keratinocyte DNA and RNA extracts. PCR assays for E1, E2, E6 and E7 DNA of CPV1 (first row), CPV3 (second row) and CPV5 (third row) 5 passages after transduction. PCR performed on RNA extract with reverse transcription (RT+), RNA extract without reverse transcription (RT-) and DNA extract (DNA). M indicating 1 kb marker with bands of about 500, 400, 350, 300 and 200 nucleotides displayed.

Table 1 Primerlist

Primer Name	Sequence	Binding & size	
COPV_no_L_SbfI_f	AAC CCT GCA GGT GTG TCA TTG ATT ACT TGT	8360 - 8389	
COPV_no_L_SbfI_r	CCC TGC AGG TGC GTA AGC GAG GGA TGT TTT	5289 - 5318	
CPV3_no_L_SbfI_f	CTT CAC CTG CAG GAC GCT CTG TTA AAA GG	7216 - 7244	
CPV3_no_L_SbfI_r	TAC CCT GCA GGA CCA TGA CAA GTC AAC	4208 - 4234	
CPV5_no_L_SbfI_f	CGC CCT GCA GGT CTA CTT CTA AAT CTA CAC GA	7229 - 7260	
CPV5_no_L_SbfI_r	GCG CCT GCA GGA TGA CAG GTC AAC AGG TGA CAA	4223 - 4255	
Nde-Sbf Oligo	TAT GAC TGC CTG CAG GCA GTC A		
COPV_E6_f	TTT GCT TGA TCT GTC GCT T	198	281
COPV_E6_r	GTT CTT TGT CCG CCT CAC T	478	
COPV_E7_f	CGC AAC CCT TTT GGA TAT T	569	280
COPV_E7_r	CTA GCC GCC ATG GTC AA	848	
COPV_E1_f	AGA CTG AGG ATG GTG GTT G	866	274
COPV_E1_r	GGC GTT AAG CTA ATG CTT G	1139	
COPV_E2_f	GCA CTG GTC ATT GCT CAG A	2665	347
COPV_E2_r	CCC AGG TGT CAA ACT CAT C	3011	
CPV5_E1_f	AGG GAG GAC AAT AGG CAG G	951	412
CPV5_E1_r	TAA ATG GCC TGG TCA ACT CC	1362	
CPV5_E6_f	ATG GAG CCT TGC AGG TCT TA	48	365
CPV5_E6_r	GCC CTC TGA CCT TTT TGA AT	412	
CPV5_E7_f	TTG GGA AAG ACG CTA CTT TGA	459	264
CPV5_E7_r	AGA CCG GAC AGC AGA ATG AT	722	
CPV5_E2_f	CTG TCA GAT CAG GTC CAG CA	2653	347
CPV5_E2_r	TCC GTG AAA TAC ACG TGG TG	2999	
CPV3_E7_f	TTG GGA AAG ACG CTA CTT TGA	444	294
CPV3_E7_r	CTC CAT GGT CAA AGC GGT AG	737	
CPV3_E6_f	GAG TGT CTC TGT GCG CGA TA	68	337
CPV3_E6_r	CAC CGC CCT CTC ACT TTT T	404	
CPV3_E1_f	AGG GAG GAC AAT AGG CAG GT	939	334
CPV3_E1_r	GGT TAG ACG CTC GCA TTA GC	1272	
CPV3_E2_f	TGC AGG ACA CCA GCT ATG AG	2840	332
CPV3_E2_r	TGT GGT GCT GGT GAC AGA TT	3171	
panAAV f	GTN AAC TGG ACY AAY GAA AAC TTT CC		
panAAV r	KKG GAA TCG CAA TGC CAA T		

Figure 1

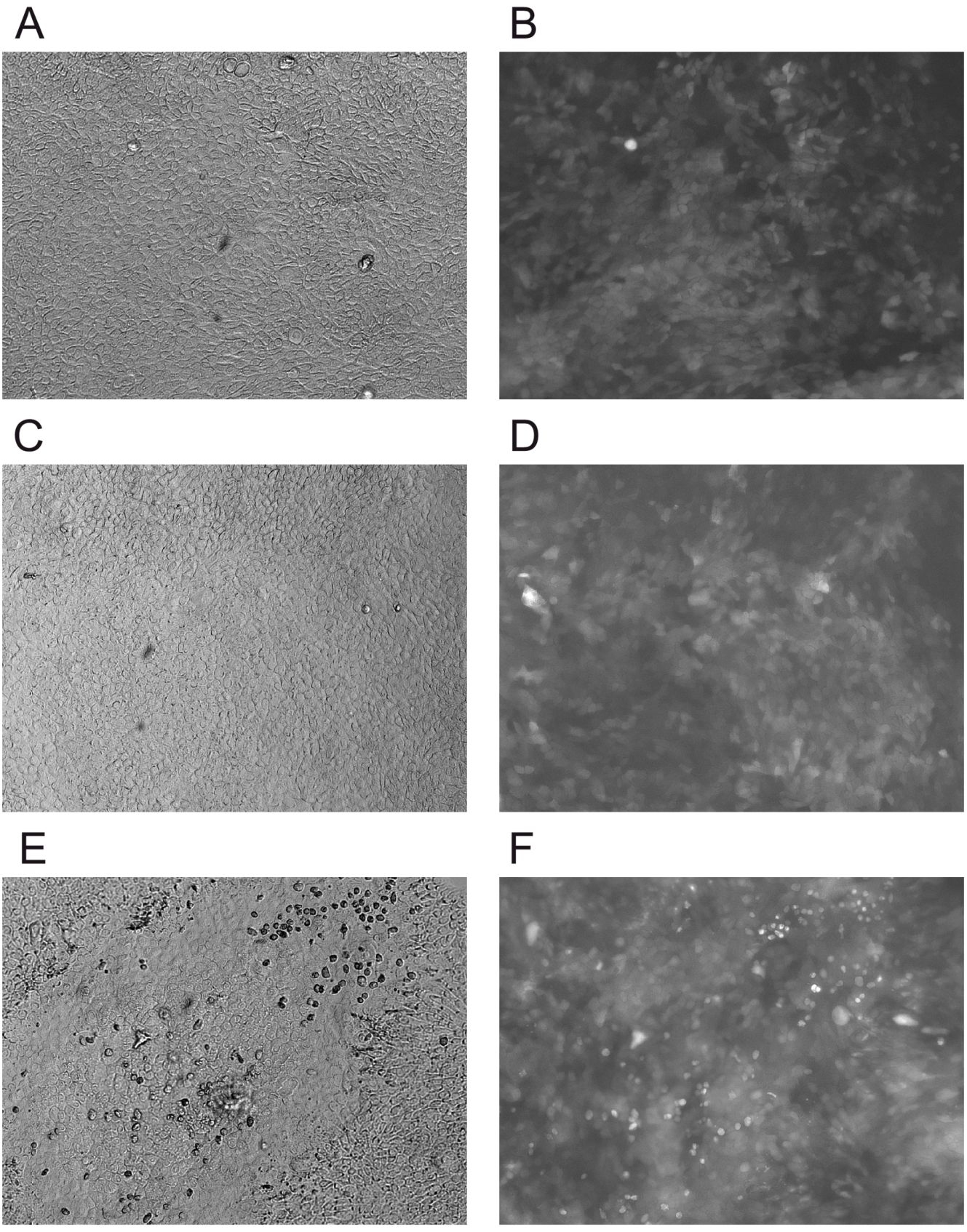


Figure 2

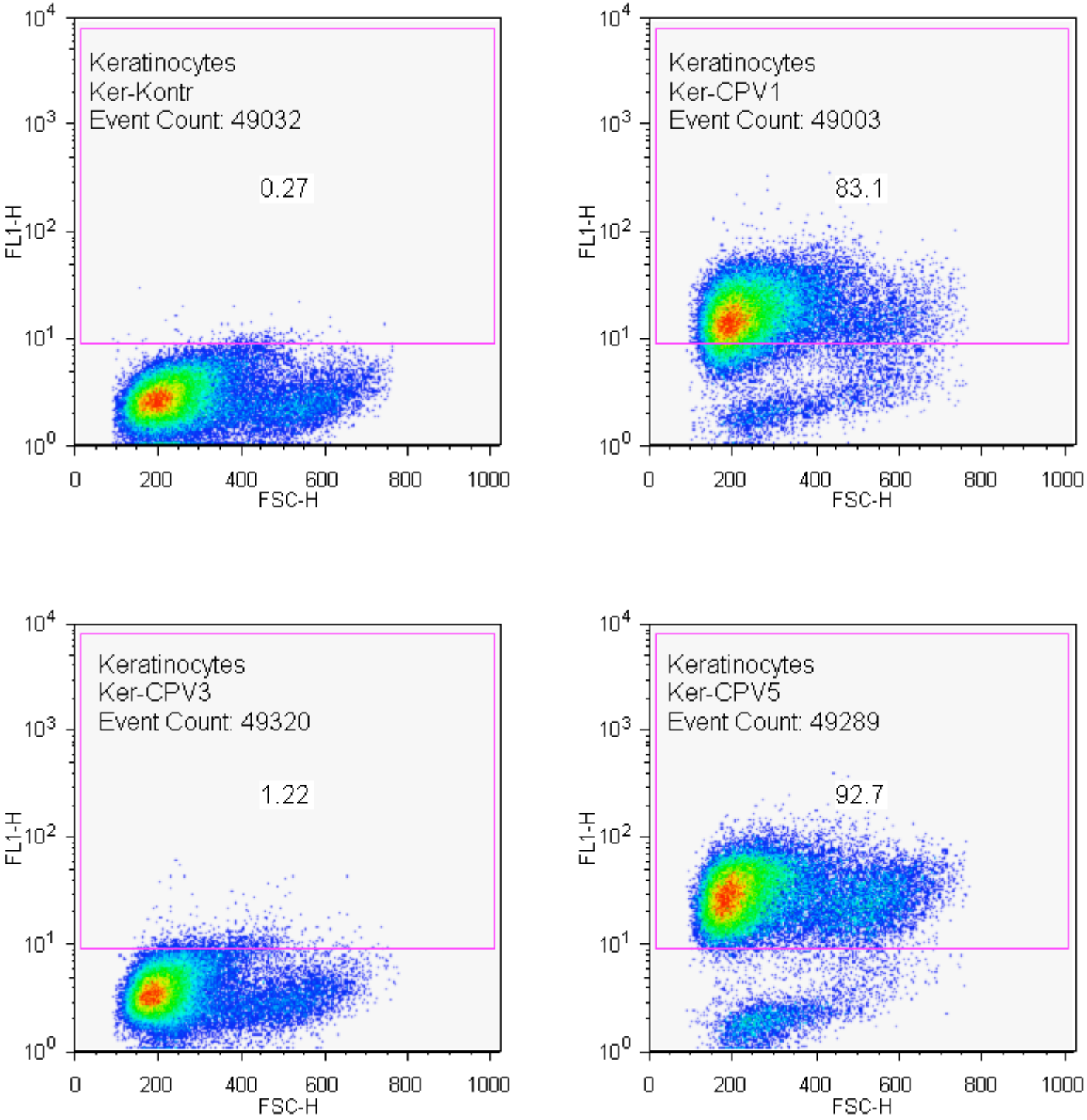
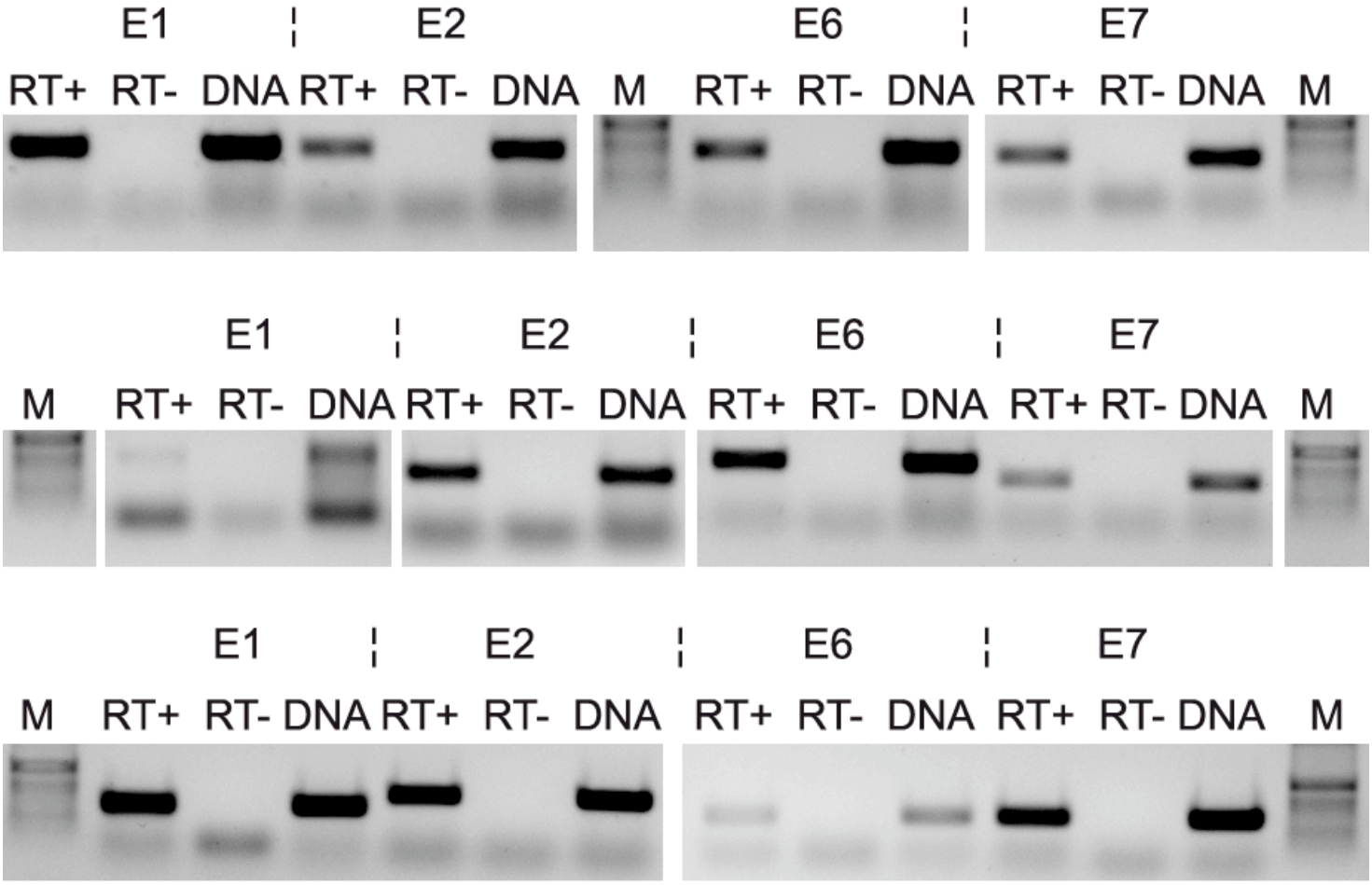

Keratinocytes
Ker-Kontr
Event Count: 49032
0.27
Keratinocytes
Ker-CPV1
Event Count: 49003
83.1
Keratinocytes
Ker-CPV3
Event Count: 49320
1.22
Keratinocytes
Ker-CPV5
Event Count: 49289
92.7
FL1-H
FSC-H
10^0
10^1
10^2
10^3
10^4
0
200
400
600
800
1000

Figure 3


E1
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Part II – Equine papillomaviruses and papillomatoses in horses

Papillomatoses in the horse are a well known problem and have already been described in ancient times. However, the best known papillomaviruses affecting horses are bovine ones (BPV1 and BPV2) involved in the equine sarcoid. This BPV1 associated semimalignant tumors mark the only confirmed pathology linked to papillomaviruses crossing a species barrier. Only one equine papillomavirus (EcPV) that is typically associated with transient benign tumours of the young horse was yet identified. Previous primarily clinical and pathological findings however suggested that there could be at least three different papillomavirus induced disorders, potentially involving different papillomaviruses.

We identified EcPVs in two other occasionally observed disorders, namely penile papillomas (EcPV2) and equine aural plaques (EcPV3). Based on the genomic sequences of these viruses a phylogenetic allocation of the two novel viral sequences was possible (*Identification of two novel equine papillomavirus sequences suggests three genera in one cluster, pages 54-60*). We established PCR and in situ hybridisation to verify whether the papillomavirus involved in the penile lesions (which are presented more often than aural plaques) and used them to test seven paraffin embedded samples from cases of the past years. All these samples were positive for either of two described variants of EcPV2. Viral DNA was located in the nuclei of koilocytes, cells typical for active viral infection in the skin (*In Situ Hybridization Supports Papillomavirus Etiology in Equine Penile Papillomas page 61-72*).

Identification of two novel equine papillomavirus sequences suggests three genera in one cluster

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Own contribution

I was substantially involved in the planning and outlining of this work. I performed all the molecular biological experiments and a major part of the data analysis. I also did the major part of the writing.

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In detail, text: Abstract by C. E. Lange and K. Tobler, introduction by C. E. Lange, materials and methods by all authors, results by C. E. Lange, discussion by C. E. Lange, C. Favrot and K. Tobler

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Research article

Identification of two novel equine papillomavirus sequences suggests three genera in one cluster

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ABSTRACT

The number of recognized papillomavirus (PV) species and potential PV genera has dramatically been increasing throughout the past decade. It seems that every host species might potentially harbour a large set of PVs, while the PVs of each species appear to belong to only a few genera.

In horses at least three conditions beside the equine sarcoid have been described, being supposedly PV induced namely classical equine papillomas, genital papillomas and aural plaques. We were able to identify the DNA of novel equine PVs (EcPVs) in the two latter disorders where PV involvement had been predicted. Both PV genomes were entirely cloned and sequenced. Both EcPV genomes, one derived from a penile papilloma, the other derived from an ear papilloma contain the characteristic open reading frames (ORFs) E6, E7, E1, E2, L2 and L1, a large non-coding region between the late and early region as well as a small non-coding region between the early and the late region. The viruses were consequently designated as EcPV2 and EcPV3.

The genomes of the three equine PVs were analysed and compared with each other and further PVs. Upon phylogenetic analyses the equine PVs group well together. Pairwise alignment of the L1 nucleotide sequences reveals that EcPV1 shares 54.9% identities with EcPV2 and 53.2% with EcPV3. EcPV2 and EcPV3 share 51.3% identities. As the three EcPVs share less than 60% of nucleotide identities in L1, they may be regarded as belonging to different genera.

1. Introduction

The family of Papillomaviridae consists of a diverse variety of papillomaviruses (PVs), characterized by a small, circular, double stranded DNA genome packaged into non-enveloped icosahedral viral particles (Howley and Lowy, 2007). Papillomaviruses have been found to be innocuous inhabitants of the healthy skin, but are also associated with various benign and malign neoplastic diseases (Howley and Lowy, 2007). While some PV types have been demonstrated to have a high oncogenic potential others are known to be less oncogenic, facultative pathogenic or believed to be apathogenic (Antonsson et al., 2000; Antonsson and Hansson, 2002; Burd, 2003; Muñoz et al., 2003). Consequently a categorization of primarily human PVs (HPVs) based on their transforming potential has been suggested, grouping the viruses into high-, intermediate- and low-risk PVs (Lorincz et al., 1992; Bosch et al., 1995). This grouping is partly reflected in the genomic characterization of the PVs. Viruses allocated to one genus based on the nucleotide sequence of the L1 open reading frame (ORF) may share some features, but the biological behaviour can differ significantly (de Villiers et al., 2004). Expanding the knowledge about the phylogenetics

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and genomic organization together with the biological effects can consequently be helpful for better understanding and characterizing of the Papillomaviridae.

To date, almost 200 distinct PVs have been cloned, sequenced, and allocated to various genera. Most of the identified PVs are HPVs, but the number of PVs found in lesional or nonlesional skin of animals is steadily growing. In horses, infections with papillomaviruses have been reported extensively in the context of the equine sarcoid (Bogaert et al., 2008, Nasir and Campo, 2008). This semi malignant neoplasia of the skin though is, as it is associated with bovine PVs, an exception, as PVs are in general believed to be species specific (Howley and Lowy, 2007). Nevertheless, at least three other potentially PV associated epithelial neoplasias have been described in horses (Scott and Miller, 2003). The so called classical equine viral papillomas are typically found in young horses. They occur mainly on the muzzle or lips but also less common in other locations (Scott and Miller, 2003). They have a transient character and were shown to be associated with EcPV1 (O'Banion et al., 1986; Ghim et al., 2004). Infrequently occurring genital papillomas in male and female horses were also linked to PVs. They appear to undergo self-regression only rarely and have been described to progress occasionally into carcinomas (Smith et al., 2009). The involvement of a genetically and serologically distinct equine PV EcPV2 has been suggested (O'Banion et al., 1986). The equine ear papillomas or aural papillomas have been described in horses of all ages (Scott and Miller, 2003). Although they have a progressive character, progression into any cancer has not been reported. However, the involvement of a distinct PV in these lesions has been suggested (Fairley and Haines, 1992; Scott and Miller, 2003).

In order to identify, genetically characterize and compare viruses involved in the three described types of equine epithelial neoplasias, viral DNA derived from a penile papilloma and one derived from an aural papilloma was amplified, cloned and sequenced. Viruses termed EcPV2 and EcPV3 were identified. The genomes of the three EcPVs were found to have certain similarities but nucleotide identities below the current definition of a genus. The suggested genetic distinctness of the viruses putatively involved in the three types of equine epithelial neoplasias could consequently be confirmed.

2. Materials and methods

2.1. Samples

A 15-year old Icelandic horse (gelding) was presented with numerous white papules on the penis and preputium. Six-millimeter-punch biopsies were taken and histological examination revealed marked irregular hyperplasia with rete ridges and hypergranulosis with clumped keratohyalin granules. A marked loss of polarity was seen in the whole epidermis, especially stratum spinosum. Koilocytes were not identified. A diagnosis of in situ carcinoma was made.

A 7-year old Haflinger horse was presented with white, well demarcated papules from both lateral face of the pinnae. Eight-millimeter-punch biopsies were taken. Histological examination was carried out and revealed mild, papillated hyperplasia of the epidermis with marked orthokeratotic hyperkeratosis and clumped keratohyalin granues. Numerous swollen keratinocytes (koilocytes) with sometimes intranuclear inclusions were also noticed. A diagnosis of papillomavirus-induced equine ear papilloma was made.

Samples for molecular-biological examination were stored at −20 °C until processing.

2.2. Amplification and cloning of genomes

Total DNA of 25 mg tissue sample was isolated using a DNeasy extraction kit (Qiagen) according to the manufacturer's recommendations. One microliter of the DNA extract was then used for RCA (Rector et al., 2004), using a TempliPhi Amplification kit (General Electrics Biosciences). Slight modifications were applied to the protocol supplied by the manufacturer: 1 μl of 10 mM dNTPs was added and the reaction time was prolonged to 16 h at 30 °C. Amplified DNA was cloned into the *Bam*HI or *Eag*I site of pBluescript II KS+ (Stratagene) in case of the isolate from the penile papilloma (EcPV2) and into the *Eco*RV, *Spe*I or the *Xba*I site in case of the isolate from the aural plaques (EcPV3) using standard procedures.

2.3. Sequence analysis

The nucleotide sequences of cloned DNA and of precipitated RCA product were determined (Microsynth) by cycle sequencing using an ABI 377 sequencer (Applied Biosystems). Positive and negative strand of one clone each were sequenced, in case of any doubts precipitated RCA product was also sequenced for verification. Manual primerwalking technique with overlapping sequences was used. The primary sequences were assembled using Contigexpress software (Vector NTI Informax).

The coding sequences for the E1, E2, L2 and L1 proteins from 50 papillomaviruses including EcPV1, EcPV2 and EcPV3 were translated and a multiple sequence alignment (MSA) for each of the four set of protein sequences was performed using MAFFT (version 6.6.11b; blossum 62 matrix, maxiterate 1000, localpair (Katoh and Toh, 2008)). Aligned nucleotide sequences were produced by matching the coding sequences with the corresponding aligned protein sequences. The four sets of aligned nucleotide sequences representing the four sets of protein sequences were combined to a single MSA by concatenating the sequences from each virus. The resulting single MSA, 9504 nucleotide positions in length, was then shortened to 4948 nucleotide positions by using GBLOCK (version 0.91b; half gap positions allowed (Castresana, 2000)). The optimal model of DNA evolution was evaluated for best fit of the dataset using MODELTEST (version 1.4.4; default settings (Posada and Crandall, 1998)). Bayesian phylogeny was inferred using MRBAYES (version 3.1.2; Markov Chain Monte Carlo with GTR substitution matrix, variable gamma rates, invariant sites, 2 runs with 4 chains of

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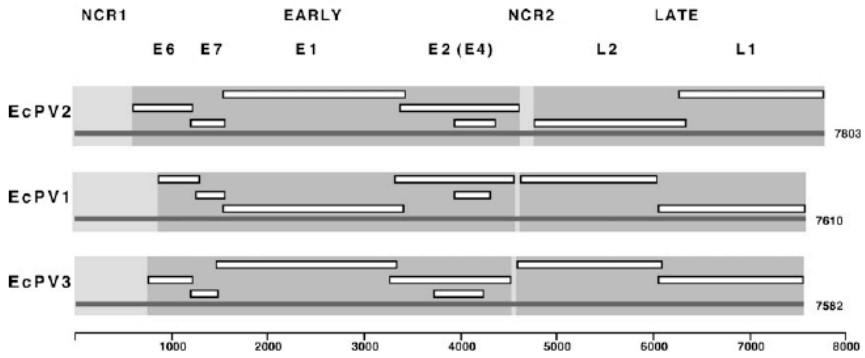

Fig. 1. Schematic presentation of the equine papillomavirus genomes and open reading frames (ORFs). Genomes are divided into sections: early genes (Early), late genes (Late) and non-coding regions (NCRs). Numbers indicate nucleotide positions. Nucleotide position number one is defined here as the first following the stop codon of the L1 ORFs.

~3,700,000 generations (Ronquist and Huelsenbeck, 2003)). Evaluation of the MCMC and final refinement of the tree were performed with BEAST (version 1.4.8 (Drummond and Rambaut, 2007)). Pairwise alignments were made in NEEDLE (EMBOSS) using the EDNAFULL matrix with a gap penalty of 10.0 and a extend penalty of 0.1. The nucleotide sequence data of the PVs were deposited in GenBank under accession numbers HM461973 (EcPV2) and GU384895 (EcPV3).

3. Results

3.1. Genome and open reading frames

The genome size of PVs ranges roughly from 7 to 8.6 kilobasepairs (kb) and a genomic organization containing typically seven or eight ORFs as well as one or two non-coding-regions (NCRs) (Howley and Lowy, 2007). The genome size of novel EcPV3 was determined as 7582 basepairs, which is similar to the other EcPVs as it is just slightly less than EcPV1 (7610), whereas the EcPV2 isolate has 7803 basepairs. The GC contents are 51% for the EcPV3 genome, 56% for EcPV2 genome, and 53% for the EcPV1 genome. In all the EcPV genomes, seven characteristic ORFs can be identified (Fig. 1). While the sizes of the ORFs encoding for the late genes appear to be quite conserved, differences can be observed among the early genes E6 and E7. The sizes of the two ORFs do not differ much in EcPV1 and EcPV3 but are larger in case of the EcPV2 isolate. The E4 ORF which usually lacks an own start codon varies also between the viruses, and therefore cannot be predicted with certainty.

3.2. Characteristic motifs

Several characteristic features on the nucleotide and amino acid level have been identified, which are predicted to play a role in the PV life cycle (Androphy et al., 1987; Wilson et al., 2002; Münger et al., 2004; Howley and Lowy, 2007). The three EcPVs were scanned to identify these putative sites and the analysis revealed typical features in the genomes of all of them. Dyad symmetry repeats

Table 1
Characteristic features on nucleotide and amino acid level.

Predicted nt-feature	EcPV1	EcPV2	EcPV3
E2 binding site (ACC-N_{5-7}-GGT)	116; 3028, 4168; 5298; 5388; 6527; 7144; 7378; 7509	318; 4828; 5193; 7591; 7760	358; 650; 1148; 4142; 4226; 5001; 6988; 7004; 7124; 7218; 7310; 7337: 7468
Dyad symmetry repeats[a] (TTGTTGTTAACAACAA)	7550 (2)	145 (3)	7510 (2)
Poly adenylation sites (AATAAA)	2255; 3749; 3756; 6882	7216	2345; 6748; 6808
SP1 binding sites (GGCGGG)	3815; 3997; 4558; 5982; 7357	4707; 4929; 5180; 6723; 7788	1915; 4272; 4311; 4798; 6967
NF1 binding sites (CGGAA)	483; 3329	2906; 3304; 3421; 3575; 3895; 5435	361; 687; 2946; 5591
AP1 binding site (TGANTCA)	3390; 4902	97	7459
TATA signals (TATAAA or TATA(A/T)A(A/T))	6049; 6051; 7600	-	1563; 6547; 6827

Predicted aa-feature	EcPV1	EcPV2	EcPV3
ATP-dependent helicase motifs in E1 (GPPNTGKS)	443aa/2016nt	455aa/2305nt	443aa/2028nt
Metal-binding motifs in E6 ($CX_2CX_{28-30}CX_2C$)	12aa/58nt; 85aa/277nt	8aa/260; 83aa/485nt	21aa/64nt; 94aa/283nt
Metal-binding motifs in E7 ($CX_2CX_{28-30}CX_2C$)	53aa/508nt	70aa/813nt	47aa/572nt
Leucine zipper domain in E1 (LX_{5-7} LX_{5-7} $LX_{5-7}L$)	–	–	264aa/1491nt
Leucine zipper domain in E2 (LX_{5-7} LX_{5-7} $LX_{5-7}L$)	–	4aa/2777nt	–

Underlined starting positions are part of the putative origin of replication.
[a] Modified in all cases in 2 or 3 positions.

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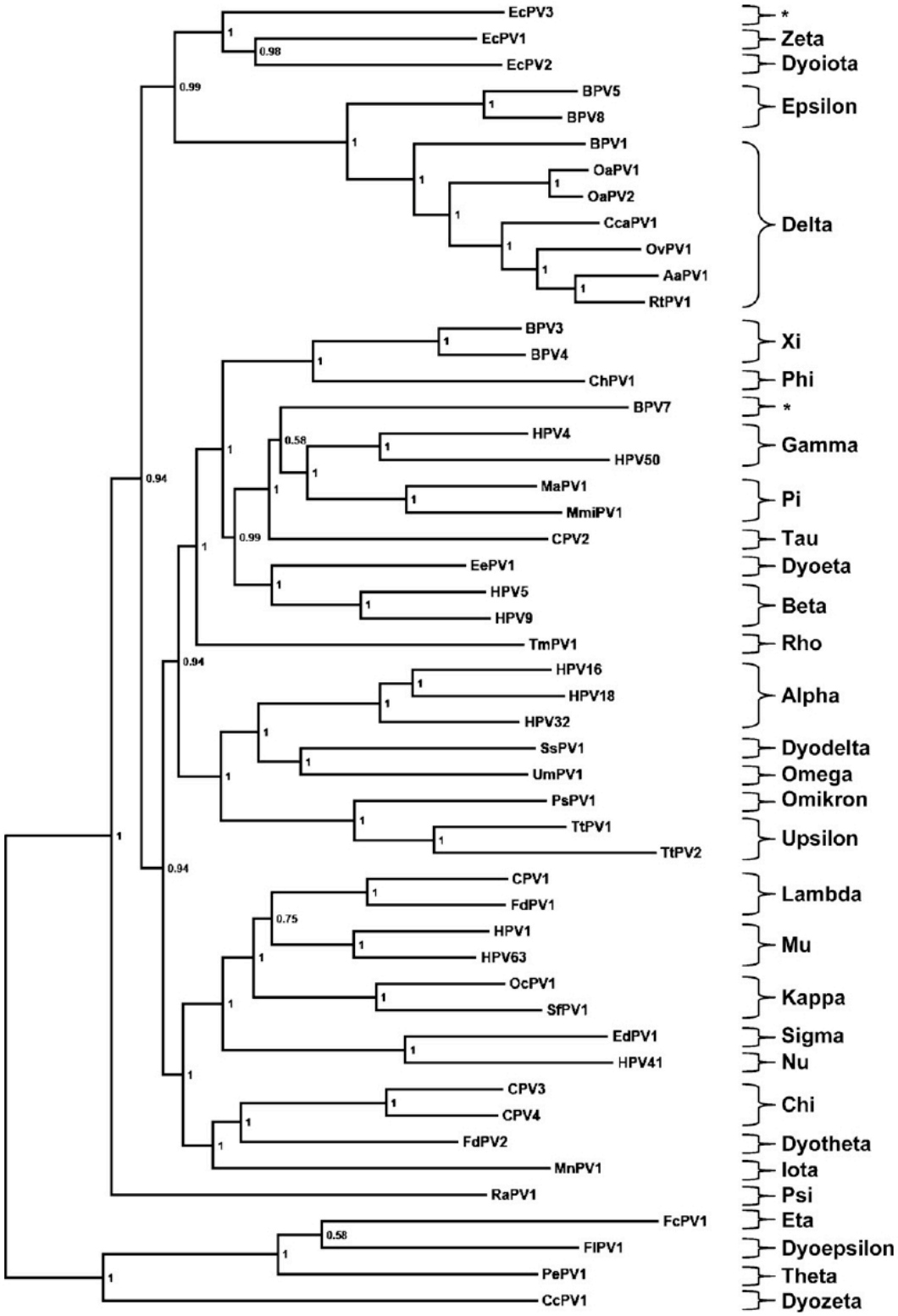

Fig. 2. Bayesian phylogenetic tree of 50 papillomaviruses (PVs). The PV-types (with GenBank accession numbers) included are: *Alces alces*: AaPV1 (M15953), bovine: BPV1 (X02346), BPV3 (NC_004197), BPV4 (X05817), BPV5 (AF457465), BPV7 (NC_007612), BPV8 (NC_009752), *Capreolus capreolus*: CcaPV1 (NC_011051), *Caretta caretta*: CcPV1 (EU493092), *Capra hircus*: ChPV1 (NC_008032), canine: CPV1 (L22695), CPV2 (AY722648), CPV3 (NC_008297), CPV4 (NC_010226), *Equus caballus*: EcPV1 (AF498323), EcPV2 (HM461973), EcPV3 (GU384895), *Erethizon dorsatum*: EdPV1 (NC_006951), *Erinaceus europaeus*: EePV1 (EF396272), *Fringilla coelebs*: FcPV1 (NC_004068), *Felis domesticus*: FdPV1 (NC_004765), FdPV2 (EU796884), *Francolinus leucoscepus*: FlPV1 (EU188799), human: HPV1 (NC_001356), HPV4 (NC_001457), HPV5 (NC_001531), HPV9 (NC_001596), HPV16 (FJ610146), HPV18 (NC_001357), HPV32 (NC_001586), HPV41 (NC_001354), HPV50 (NC_001691), HPV63 (NC_001458), *Mesocricetus auratus*: MaPV1 (E15111), *Micromys minutus*: MmiPV1

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flanked by putative E2 binding sites, several more putative E2 binding sites and at least one poly adenylation signal are present in every EcPV (Table 1). SP1, NF1 and AP1 binding sites could be predicted in all three EcPVs but a classical TATA box is missing in EcPV2 (Table 1). Examination of the putative protein sequences reveals an ATP-dependent helicase motif located in each E1 and two or one metal-binding motifs in E6 and E7, respectively (Table 1). No putative pRB binding site (LXCXE) could be identified in any of the EcPV E7 amino acid sequences.

3.3. Sequence analysis and comparison

In order to possibly allocate the novel PV, phylogeny based on the aligned E1–E2–L2–L1 sequences was determined. Sequences of 50 PVs, representing all presently classified genera including those of the three EcPVs were aligned (Fig. 2). Based on the resulting tree, the three viruses originally extracted from horses EcPV1, EcPV2, and EcPV3 were found to cluster together. They were allocated in relative vicinity of Chi, Dyotheta, Rho and Psi PV genera.

In order to investigate the relatedness of the novel PV isolates according to the present guidelines of PV classification (de Villiers et al., 2004; Bernard et al., 2010), pairwise alignments were performed based on the nucleotide sequences. In case of the L1 ORF, which is currently used for classification, EcPV1 was found to share 54.9% identities with the EcPV2 isolate and 53.2% with EcPV3, while the EcPV2 isolate shares 51.3% identities with EcPV3. EcPV1 and EcPV2 are therefore the closest to each other among the tested sequences, while the closest known relative to EcPV3 is the sequence of EcPV1. The other ORFs of the EcPVs were also compared by pairwise alignments. In case of E1, as the second ORF being relatively conserved, EcPV1 was found to share 53.4% identities with the EcPV2 isolate and 56% with EcPV3, the EcPV2 isolate and EcPV3 share 48.8% identities with each other. Among the other ORFs, identities between EcPVs were all between 39.5% and 46.4%.

3.4. Associated clinical conditions

The association of PVs with certain clinical signs is of special interest. The sequence of EcPV1 has been obtained from cutaneous papillomas affecting the muzzle of horses and is characterized as being typically associated with transient papillomas in horses younger than 3 years of age (O'Banion et al., 1986; Scott and Miller, 2003). The sequence of EcPV2 was isolated from a penile papilloma and has repeatedly been detected in genital neoplasias (unpublished data). EcPV3 as described here was found in aural plaques of a horse. The three mentioned clinical conditions have been referred to be equine epithelial neoplasias in which a papillomavirus involvement had been previously demonstrated or suggested (Scott and Miller, 2003).

4. Discussion

PVs have been found in a large genetic variety in humans and in increasing numbers in various other mammalian species. Their association with various diseases was demonstrated or suggested and certain types of PVs seem to be responsible for distinct pathologies. In horses, three epithelial neoplasias have been described and the question of PV involvement was previously addressed by various methods (O'Banion et al., 1986; Fairley and Haines, 1992; Ghim et al., 2004; Smith et al., 2009). It was concluded from the results, that classical equine viral papillomas, genital papillomas and equine ear papillomas are induced by different PVs (Scott and Miller, 2003). As the classical equine viral papilloma associated EcPV1 was so far the only equine PV characterized in more detail, our focus was on genital papillomas and equine ear papillomas. The presented finding of DNA belonging to two distinct PVs other than EcPV1 supports the hypothesis of three completely independent pathologies. Only one case involving each PV was included here, so further research is needed to corroborate a potential causative association between the viruses and the diseases. However, we have detected by PCR EcPV2 DNA in association with numerous cases of penile papilloma (data not shown), which supports the association with EcPV2 with this condition. Unfortunately, no further cases of aural papillomas were available for analyses. Although further evidence will be needed, this does not eliminate the fact that a thus far not described PV has been detected in the context of this condition. The distinct nucleotide sequences of the three EcPVs goes in line with the difference in the supposed course of infection leading to transient infections in young animals in case of EcPV1, or to persistent or even progressing lesions on the genital mucous membranes in case of EcPV2 and at the ear in case of EcPV3. The precise mechanisms remain nevertheless unclear at this point. An important set of experiments to address this issue will include analysis of viral gene expression within the lesions. However, either RNA-preserving samples or specific antibodies against the viral gene products should be available for that purpose.

The genomic organization of the three EcPVs is relatively similar containing the classical ORFs E6, E7, E1, E2, E4, L2 and L1 as well as a large NCR. The length of the individual ORFs was not found to vary significantly, except for E7 and to less extend E6. In case of EcPV2 we decided to annotate the E6 by using the second start codon as putative start. We did so because alignments with other E6 sequences did not reveal any homologies in first part of the putative long E6 ORF.

Comparison of the novel sequences with the GenBank database revealed that a sequence of an EcPV2 already

(DQ269468), *Mastomys natalensis*: MnPV1 (NC_001605), *Ovis aries*: OaPV1 (NC_001789), OaPV2 (U83595), *Oryctolagus cuniculus*: OcPV1 (NC_002232), *Odocoileus virginianus*: OvPV1 (NC_001523), *Psittacus erithacus*: PePV1 (NC_003973), *Phocoena spinipinnis*: PsPV1 (NC_003348), *Rousettus aeyptiacus*: RaPV1 (NC_008298), *Rangifer tarandus*: RtPV1 (AF443292), *Sylvilagus floridanus*: SfPV1 (NC_001541), *Sus scrofa*: SsPV1 (NC_011280), *Trichechus manatus*: TmPV1 (NC_006563), *Tursiops truncatus*: TtPV1 (EU240894), TtPV2 (NC008184), *Ursus maritimus*: UmPV1 (NC_010739). Numbers at internal nodes represent the posterior probability support values. Unclassified PVs are marked with an asterisk.

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exists in the database, supposedly deriving also from a genital neoplasia in a horse (NC_012123). This sequence is one nucleotide shorter, and annotated in a slightly different way. The major difference between our sequence and this published one is the E1 ORF which is significantly longer in case of the novel isolate.

The alignment of the L1 ORF has revealed identities clearly below 60%, allocating each of the three EcPVs to a separate genus, *Zeta* and *Dyoiota* in case of EcPV1 and EcPV2 while EcPV3 remains yet unclassified. As the novel isolate of EcPV2 shares 98.8% identities with the sequence present in GeneBank in the L1 ORF, we may, according to the guidelines for classification (de Villiers et al., 2004), be facing variants of the same virus. In a strict sense, L1 is perceived as the single most important gene to be used for PV classification (de Villiers et al., 2004; Bernard et al., 2010). However, it has been shown that it is of advantage to include the conserved regions of further ORFs into the phylogenetic analysis, especially when relatively distant PVs are to be compared (Garcia-Vallve et al., 2005; Erdélyi et al., 2008; Bennett et al., 2010). Therefore, we chose to use the latter strategy for generating the phylogenetic tree presented here.

It is interesting to find the three EcPVs to cluster quite well upon phylogenetic analysis, while the PV genera of other species appear rather scattered. The most likely explanation is though, that we still have far too few PV sequences available to elucidate the phylogenetic relations being well supported over all supertaxa and genera.

The finding of the novel EcPVs nevertheless helps to improve our understanding of PV phylogeny on the one hand and our interpretation of described pathologies on the other hand. More details about the epidemiology and the molecular mechanisms of the discovered and yet uncovered EcPVs will be needed to clarify our picture about PV associated epithelial neoplasias.

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In Situ Hybridization Supports Papillomavirus Etiology in Equine Penile Papillomas

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Own contribution

I was substantially involved in the planning and outlining of this work. I performed all the molecular biological experiments including hybridisations and a major part of the data analysis. I also did the major part of the writing.

In detail, figures: Figures 1, 5, 6, 7 and 8 by C. E. Lange, figures 2 and 3 by P. Grest, figure 4 by C. Favrot

In detail, text: Abstract by C. E. Lange and C. Favrot, introduction by C. E. Lange and C. Favrot, materials and methods by all authors, results by C. E. Lange, C. Favrot and P. Grest, discussion by C. E. Lange and C. Favrot

In Situ Hybridization Supports Papillomavirus Etiology in Equine Penile Papillomas

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Abstract

Equine penile papillomas, in situ carcinomas, and invasive carcinomas are suspected to belong to a continuum of papillomavirus-induced diseases and to represent the equine counterpart of human bowenoid papulosis. They present clinically as grayish small plaques to flat warts developing subsequently into more hyperplastic or, alternatively, ulcerated lesions. Histologically, lesions range from hyperplasia of the epidermis with sparse koilocytes to in situ or invasive squamous cell carcinomas.

In order to test the hypothesis that these lesions are papillomavirus-induced, seven samples of horses with characteristic clinical and histological findings of penile papillomatosis and/or squamous cell carcinomas were collected and tested by PCR. The respective primers were designed to amplify DNA of the recently discovered equine papillomavirus EcPV2. The tested samples were all found positive. To further support the findings and to locate the papillomavirus DNA an in situ hybridization for the detection of EcPV2 DNA was established. The samples tested by this technique were found to harbor a vast amount of papillomavirus nucleic acid concentrated in the nucleus of the koilocytes. These findings taken together support the theory of papillomaviruses inducing the described penile lesions in horses.

Keywords

horse, in situ hybridization, in situ squamous cell carcinoma, invasive squamous cell carcinoma, Papillomavirus, PCR, penile papilloma, penis

Papillomaviruses (PVs) are small double stranded DNA viruses that play an important role in the development of various benign and malignant neoplastic disorders of the skin and mucous membranes in humans and animals.[5] In horses, lesions being associated with bovine PVs, namely the equine sarcoid, have been well described.[1,7] This phenomenon however, the interspecies transmission of PVs, is regarded as rather exceptional. Three actual equine PVs (EcPVs) have been identified in lesions that had previously been suggested to be potentially PV associated.[4,6,8] Those are the so called classical equine viral papillomas which are typically found in young horses, genital papillomas, and equine aural plaques.[6] One of the viruses, namely EcPV2, has not only been identified in genital papillomas and carcinomas from which it had been isolated, but also in ocular squamous cell carcinomas.[9] Additionally, EcPV2 DNA was identified in swaps of clinically unaffected skin.[9]
Equine penile papillomas, in situ carcinomas, and invasive carcinomas are suspected to represent the equine counterpart of human bowenoid papulosis. They present clinically as greyish small plaques to flat warts developing subsequently into more hyperplastic or, alternatively, ulcerated lesions. Histologically, lesions range from hyperplasia of the epidermis with sparse koilocytes to in situ or invasive squamous cell carcinomas.
The finding of PV DNA on unaffected skin rises the question of what role the virus plays in the PV positive lesions. To address this question we evaluated two PCRs and a specific in situ hybridization (ISH) and applied the techniques on samples of penile papilloma and penile SCC.

Materials and Methods

Samples

Samples were obtained from the Institute of Veterinary Pathology and were chosen according to the original clinical and pathological diagnosis.

In situ hybridization

To generate an ISH probe, forward (5`- GAG CTG TGC AGT GTC ACG TT -3`) and reverse (5`- TCT CCT GGA CAA GCC ACT CT -3`) primer were designed based on the L1 gene sequence of the published sequence of EcPV2. The DIG labeled probe was generated using the PCR DIG Probe Synthesis Kit (Roche) according to the manufacturer's protocol. Following PCR conditions were applied: 95°C for 3 min followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Labeled PCR products were extracted from an agarose gel using a MinElute gel extraction kit (Qiagen).

A DIG labelled probe targeting the GFP gene was generated in an analogous way employing following forward (5`-AGG ACG ACG GCA ACT ACA AG-3`) and reverse (5`-CTG GGT GCT CAG GTA GTG GT-3`) primers. The same PCR conditions were used, a pHSV-eGFP amplicon vector served as template.[2] This probe served as negative control throughout the hybridization experiments.

Tissue sections (3-4µm) were obtained using a microtome (HYRAX M55, Zeiss) and samples were placed onto positively charged slides (Thermo Scientific). Sections were dewaxed (3 x 5 min in Xylol, followed by 5 min in Isopropanol, 5 min in 96% Ethanol, 5 min in 100% Ethanol and finally 5 minutes in sterile water).

To inactivate endogenous phosphatases, the slides were washed twice for 5 min with PBS before and after incubation in 0.2 M HCl for 8 min. The tissue was permeabilized by treatment with 10µg/ml proteinase K in 10 mM Tris-HCl (pH 7.5) for 10 min followed by two washing steps in PBS for 10 min and equilibration in 2 x SSC for 2 min. Denaturation of the DNA within tissue sections was performed by adding 120 µl of denaturation buffer (70% formamide, 2 x SSC), covering the sections with a coverslip and incubation of the slide for 90 sec at 80°C. Thereafter, the slides were immediately submerged in 70%, 90% and 100% of ice cold ethanol for 3 min each and then air dried.

Thirty ng of probe were denatured in 50 µl hybridization solution (50 % deionized formamide, 2 x SSC, 1 x Denhardt`s solution, 10 % dextran sulfate, 0.5 mg/ml yeast tRNA, 0.5 mg/ml hydrolyzed salmon sperm DNA), for 10 min at 80°C before they were applied to the slides.

Slides were cover slipped and hybridized at 55°C for 20 h in a humid chamber.

After hybridisation slides were washed with 2 x SSC for 10 min followed by 2 x 50% formamide, 1 x SSC and one with 0.2% SSC each one for 20 min at hybridization temperature. For color detection, the slides were washed 3 x 5 min in TNT buffer (0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl, 0.05% Tween 20), incubated for 30 min in TNB (0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl, 0.5% blocking reagent

(Roche)) followed by 300 µl per slide antiDIG-AP Fap antibody (1:500 Roche) in TNB at RT for one hour in a humid chamber. After 3 washing steps with TNT for 5 min, followed by a 2 min equilibration step in detection buffer (100 mM NaCl, 100 mM Tris-HCl, pH 9.5; 50 mM $MgCl_2$), the slides were incubated with substrate solution (337 µg/ml NBT (Roche), 175 µg/ml BCIP (Roche), 5 mM Levamisole in detection buffer) for 2 hours. Slides were counterstained for 4 min with nuclear fast red and finally rinsed in water for 5 min. Stained slides were fixed with Kaiser's glycerol gelatin. If not indicated otherwise, all steps were performed at room temperature.

PCR evaluation

Two primer combinations were evaluated for PCR. One was the primer combination EcPV2probe f / EcPV2probe r which amplifies a 294nt stretch of the L1 open reading frame of EcPV2 and served also to generate the ISH probe. The second one was the primer combination EcPV2d f (5`- CAG ACT TGT CTG GGC TCT CC -3`) and EcPV2d r (5`- TCC CGC CTA GCA TAG AAG AA -3`), which had been designed to amplify a 474nt stretch of the L1 open reading frame of EcPV2. To determine the approximate detection levels of the PCRs serial dilutions were made from 10^9 down to 10^1 molecules of an entire genomic clone of EcPV2. For that purpose the DNA concentration of the plasmid stock solution was measured using a Spectrophotometer (ND-1000, Thermo Scientific). According the plasmid-size the amount of molecules per microliter was calculated. RedTaq ready reaction mix (Sigma) was used and conditions were chosen for both PCRs as follows: 94°C for 3 min followed by 39 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec.

DNA amplification and analysis

To test the samples total DNA of fixed tissue samples was isolated using a DNeasy extraction kit (Qiagen) according to the manufacturer's recommendations for paraffin embedded tissues. EcPV2probe f / EcPV2probe r PCR was used to test the samples. Electrophoresis in a 1% agarose gel containing ethidium bromide was used to detect the amplified fragments. PCR products were extracted from the gel using QIAEX II DNA extraction kit (Qiagen).

The nucleotide sequences of the PCR products were determined (Microsynth) by cycle sequencing using an ABI 377 sequencer (Applied Biosystems). The sequences of each isolate were aligned and a consensus sequence of 169 nucleotides was generated for each one. The sequences were compared

with the NCBI database (BLAST X) and were all aligned with the according L1 stretches of the two available sequences of EcPV2.

Results

Histology and ISH

Seven male horses (six geldings, one unknown) with depigmented plaques, nodules and/or ulcerations of the penis were included in the study. Their ages ranged from 8 to 23 years; three patients were Islandic horses (cases Nos. 4, 6 and 7) (Fig. 1), one Noriker horse (case No. 5), one Westphalian warmblood (case No. 1), one cob (case No. 2), and one horse of unknown breed (case No. 3).

Tissue biopsies were taken for histological examination, processed routinely and Hematoxylin & Eosin stained. In all seven cases hyperplastic areas were present, displaying regular differentiation of the epidermis with large irregular rete ridges formation, and containing scattered degenerated cells with homogeneous pale cytoplasm as well as scattered koilocytes (Figs. 2 & 3).

In some cases, namely cases Nos. 3, 5 and 6, there were also areas with progression to in situ squamous cell carcinoma characterized by irregular differentiation, inhomogeneous nuclear sizes in the different levels of the epidermis and in some cases mitoses above the basal cell layer. In other ones, namely cases Nos. 1, 2, 4 and 7, a diagnosis of squamous cell carcinoma was made based on the finding of invasion of the underlying dermis and numerous neoplastic cells in these areas koilocytes were less frequent in the overlying epidermis (Fig. 4). However, in the latter cases in situ squamous cell carcinomas could also be found in other locations. One case of each of the groups was chosen for ISH analysis, using the EcPV2 as well as the GFP control probe.

Upon ISH a strong signal was evident in many of the koilocytes, while no signal was present when a control probe was used (Figs. 5-6).

DNA amplification and analysis

In order to use PCR for PV detection, two primer combinations amplifying DNA from the L1 open reading frame of EcPV2 were evaluated. Both combinations performed equally well when tested on serial dilutions of EcPV2-plasmid DNA (Fig. 7). The detection level in both cases was around 100 copies of template. However, as fixed samples were used in this study, the primer combination with

the smaller product was chosen. DNA could be amplified from all seven samples (Fig. 7). The sequencing of the products confirmed the DNA to belong to EcPV2. While the sequences obtained from cases Nos. 1, 2, 3 and 5 were identical, those of the cases Nos. 4, 6 and 7 differed in two positions (6921 and 7002 relative to EcPV2-HM461973). Aligning the seven sequences with the according stretches of the two EcPV2 sequences revealed that the sequences of cases Nos. 1, 2, 3 and 5 were identical with EcPV2-NC_012123 while the sequences of cases Nos. 4, 6 and 7 were identical with EcPV2-HM461973 (Fig. 8). In cases were more than one sample of a horse was tested the same variant was consistently detected.

Discussion

Equine penile papillomas, in situ carcinomas, and invasive carcinomas are a common disease and may require harsh treatment (amputation) when diagnosed in late stages. The involvement of PVs had been suggested and a putatively involved PV had been identified. However, although good evidence exists that EcPV2 is causally involved in the mentioned pathologies, the finding of DNA in unaffected skin makes further evidence desirable.[6,9] The histological findings of the cases included here support the previous hypothesis, as characteristics of a viral infection like koilocytes are present. To confirm and determine the viral DNA in fixed samples, PCR is the most feasible method. Therefore, we decided to design primers binding in the L1 ORF as this is the most conserved region in the papillomavirus genome, and it is also used for classification.[3] The evaluation of the two primer combinations revealed almost equal sensitivity tested on plasmidic DNA as template. We chose the primer combination amplifying the shorter stretch of DNA, as the samples we had for testing were extracted from formalin fixed, paraffin embedded tissues. Since formalin is known to induce DNA damage, the chances of successfully amplifying DNA from such a sample is higher if the PCR product is small. Additional evidence from own experiments encouraged that choice (data not shown). The chosen PCR amplified DNA from all tested samples. Sequencing of the bands confirmed the DNA to be identical with EcPV2. Interestingly, the short stretches of sequences of some of the samples corresponded exactly to one of the known EcPV2 variant while the others exactly to the other EcPV2 variant. The sample size of this study is of course too small to draw definite conclusions from this, but both can obviously be found in the penile lesions.

ISH was established to determine whether the EcPV2 DNA, which had been amplified from the samples, is indeed located within the cells, indicting a causal involvement, or if it is just present on the

skin in the role of a bystander. For this purpose, an EcPV2 specific and an unrelated probe of about equal size were generated and applied in the same way. Signal was only present in the sample when EcPV2 probe was applied. As DNA was detected within the koilocytes, it can be ruled out that the DNA is only present superficially on the skin. As koilocytes are regarded as typical indicators for a PV infection, finding PV DNA within their nucleus supports the hypothesis that EcPV2 is involved in the development of the respective lesions.

In conclusion, a PCR suitable for EcPV2 detection in fixed samples has been evaluated and successfully applied. DNA of two EcPV2 variants was found in seven samples of equine penile papilloma, in situ and/or invasive carcinoma. The association of viral DNA with koilocytes has been shown by ISH. These findings taken together support a causal context of equine penile papilloma, in situ carcinoma, and invasive carcinoma with EcPV2.

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Declaration of conflicting interests

The authors declare that they have no conflicts of interest with respect to their authorship or the publication of this article.

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Figure Legends

Figure 1

Penis; horse, case No. 7. Amputated distal part of a penis with neoplasias.

Figure 2

Penis; horse, case No. 6. Hyperplastic epidermis containing scattered degenerated cells with homogeneous pale cytoplasm and koilocytes with vacuolated cytoplasm. 10x objective, HE stain.

Figure 3

Penis; horse, case No. 1. Numerous koilocytes within hyperplastic epidermis. 40x objective, HE stain.

Figure 4

Penis; horse, case No. 1. Infiltratively growing squamous cell carcinoma, 4x objective, HE stain.

Figure 5

Penis; horse, case No. 1. Epidermal hyperplasia with numerous koilocytes. 10x objective, in situ hybridization of DIG labeled DNA probe complementary to GFP, counterstain with nuclear fast red.

Figure 6

Penis; horse, case No. 1. Epidermal hyperplasia with numerous koilocytes. 10x objective, in situ hybridization of DIG labeled DNA probe complementary to EcPV2, counterstain with nuclear fast red.

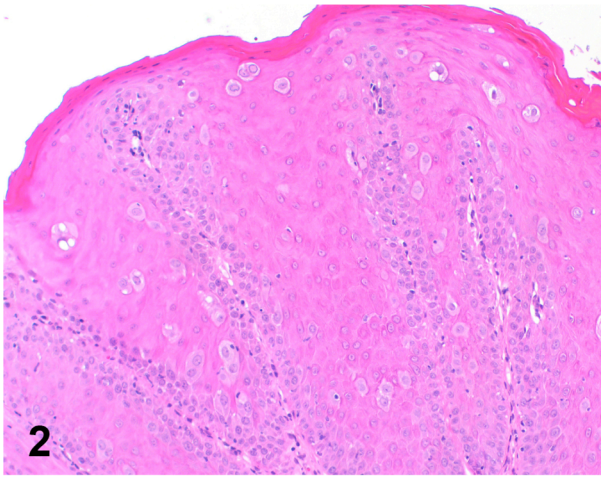
Figure 7

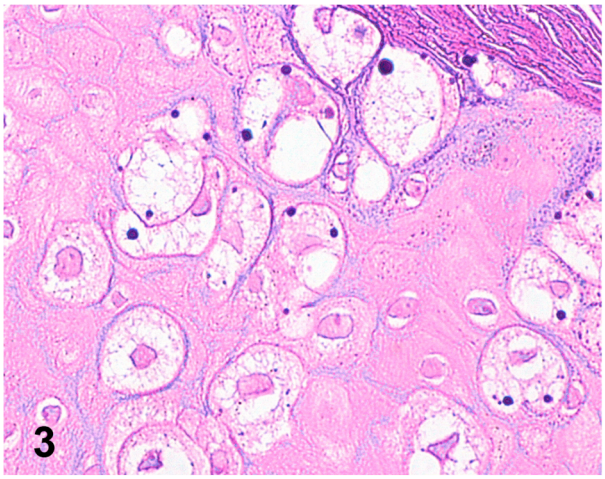
PCR evaluation of the primer combinations EcPV2d (A) and EcPV2probe (B) indicating the target molecules in the test solution. PCR of the seven equine samples using the primer combination EcPV2probe (C). M stands for 1kb marker.

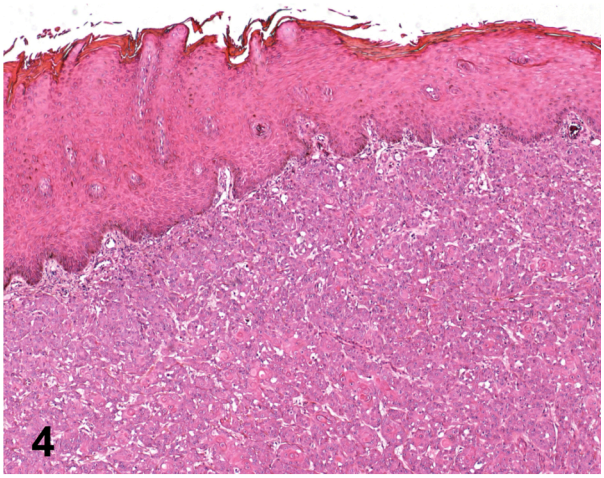
Figure 8

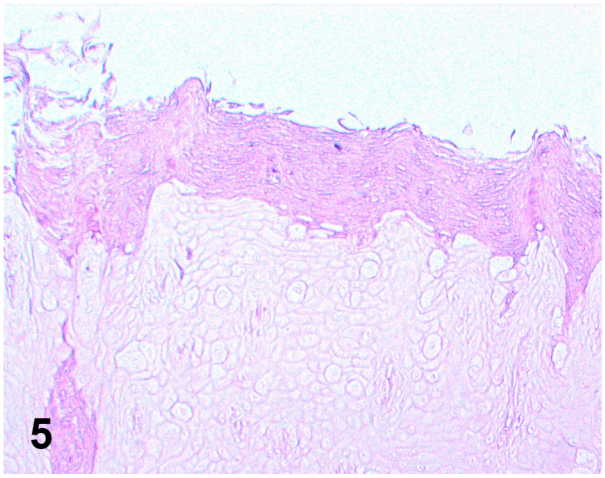
Partial electropherograms from the PCR products of the seven samples indicating the two differing bases. Positions relative to the sequence of EcPV2-HM461973.


1


2


3


4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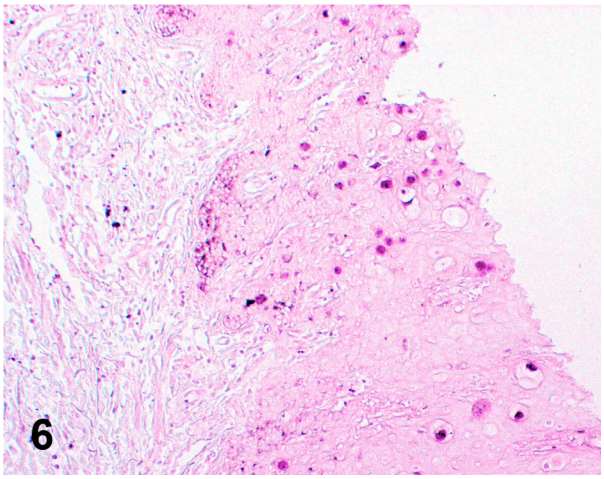
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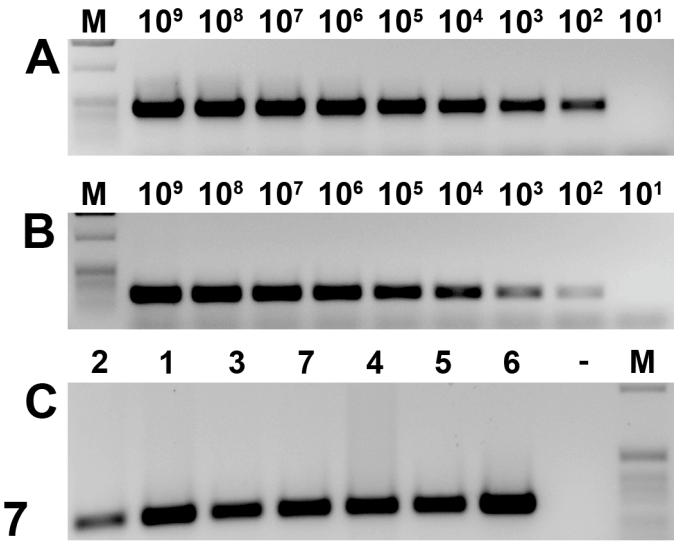
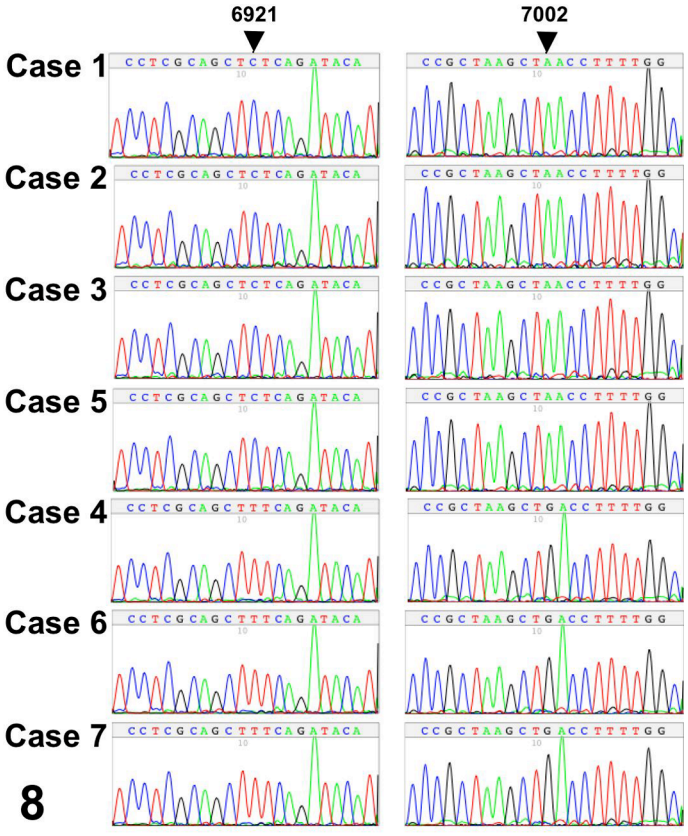

A
M 10⁹ 10⁸ 10⁷ 10⁶ 10⁵ 10⁴ 10³ 10² 10¹
B
M 10⁹ 10⁸ 10⁷ 10⁶ 10⁵ 10⁴ 10³ 10² 10¹
C
2 1 3 7 4 5 6 - M
7


6921
7002
Case 1
Case 2
Case 3
Case 5
Case 4
Case 6
Case 7
8

Conclusions and Outlook

Much has been found out about papillomaviruses, the associated diseases and the biology of the viruses affecting humans. Far less is known about the situation in most animal species and about the phylogeny of the *Papillomaviridae* family as such. Consequently there is still a need to learn more about papillomaviruses, for reasons of animal welfare and conservation but also for a better understanding of mechanism of virus biology and evolution.

As described, several experiments were made in order to expand our knowledge about papillomas and papillomaviruses in dogs and horses. In dogs we identified four distinct conditions of endophytic papillomas that were associated with the DNA of different papillomavirus types (pages 26-31). Two of these types, namely CPV1 (COPV) and CPV2 were previously described (Delius, 1994; Yuan, 2007), the other two were not. As the association of certain types of papillomaviruses with certain lesions has been described in humans, the findings indicate some parallels (Arndt, 1994; Beck, 1995). While CPV2 has previously been reported to be involved in the same ones as described in our case there remains a desire to assess whether the other papillomavirus will consistently been found in the reported lesions in the future (Goldschmidt, 2006). Nevertheless, as such parallels have been anticipated this supports the validity of our approach to identify putative papillomavirus associated skin disorders in animals by inferences from known human ones. Besides from testing whether the associations suggested will consistently be found it may as well be worth looking for the subtypes and variants involved in the inverted papillomas. It is currently a field of intense research, what influence variations in the early genes E6 and E7 have on the pathologies associated with HPVs (Xi, 2006). Similarly it could be of interest to test whether there are for example differences between CPV1 isolates found in oral papillomas and those CPV1 isolates found in skin lesions like inverted papillomas.

In order to characterize the agents involved in papillomavirus associated disorders on the level of their genetics and phylogenetics, one of the above mentioned and two other novel canine papillomavirus genomes were cloned, and analysed (pages 32-41). A consequent comparison of all canine papillomaviruses supported not just previous suggestions about dogs being affected by their own set of papillomaviruses but indicated also that these viruses, similar to what is known about human and to a lesser extend about bovine papillomaviruses, cluster together in only a few phylogenetic genera (de Villiers, 2004). Based on the data, though

limited, it may be inferred, that some predictions about the course of infection are possible based on viral genetics in combination with clinical findings. Detailed clinical assessment of papillomatoses and sequencing of further canine papillomaviruses can consequently be very helpful to test the scheme we indicated and to expand it. In the future it may help to predict the course of infection if it may turn out, that certain types or clades of CPVs are typically associated with transient and others with persistent disorders. In this context it will for example be interesting if putatively novel Chi papillomaviruses will be associated with pigmented plaques and vice versa.

Although it is well established that papillomaviruses are responsible for several benign and malignant disorders, their DNA has also been found on the clinically healthy skin of humans and several other species (Antonsson, 2000; Antonsson, 2003). To be able to put our previous findings of certain papillomaviruses being associated with certain disorders into a broader context and to be able to test dogs with well established methods for papillomavirus DNA detection, eight broad range PCR assays were tested for their validity in the context of canine papillomaviruses (pages 42-48). The most suitable PCR assay was chosen to test samples of clinically healthy dogs for papillomavirus DNA. Finding more than 50% of the dogs PCR positive indicates a high prevalence of papillomaviruses among the dog population, although none of tested animals displayed any signs of clinical papillomatosis. Similar prevalences can be found in humans (Antonsson, 2000). It remains an open question whether these dogs do carry papillomaviruses or just their DNA superficially on their skin or suffer from subclinical infections. They may as well be protected by antibodies, however the seroprevalence against the most abundant CPV1 was in a very similar population determined to be only around 10% (Lange, 2008). While the attempt in this study was to choose and use primers with a broad range, establishing a sensitive PCR based assay to able to distinguish beetween the different CPVs without the need for sequencing could be a desirable alternative approach for the future. Thus the distribution of individual CPVs could be determined and infections with more than one CPV could be identified. Such an approach would greatly benefit from a large number of entirely sequences CPV genomes. Furthermore it might be very informative to test for antibodies directed against CPVs and for CPV DNA in parallel to correlate such findings and to possibly infer the relevance of CPV DNA on the dogs skin. In this context studies testing individual dogs repeatedly over a certain period of time would be most informative, and could greatly improve our knowledge about papillomavirus biology and epidemiology.

A major and challenging question that arises from all these results is what the underlying mechanisms of papillomavirus associated disorders are. As model systems for bovine and human papillomaviruses have in the past provided valuable insights, a similar approach was projected (Baker, 1987; Fehrmann, 2005). To have such a tool for studies about the biology of canine papillomavirus infections, dog keratinocyte cell lines containing CPV DNA were established (pages 49-65). As the cell lines have been shown to harbour the papillomavirus DNA over more than a hundred passages they seem to be suitable for various experimental approaches. These cell lines can for example be used to address questions about the effects of the papillomavirus DNA and their gene products on the host cells using quantitative PCR or microarray techniques. Several genes have already been identified, who are typically up or down regulated in case of papillomavirus infections in humans. Some of those, like p16INK4a have a high predictive value about the malignant potential of a papillomavirus infection (Grce, 2010). RAFT cultures can in this context be a precious tool as it is an experimental setting close to the natural environment of papillomaviruses. As the papillomavirus lifecycle is closely linked to that of the host cell in such a setting cell stages exist, that are absent in monolayer cultures. Thus the introduced CPV DNA may unveil its potential only in such a culture system.

The cell lines make it also possible to test the effects of cofactors which are often involved in malignant papillomavirus lesions, at least to a certain extend (Howley, 2007). Putative cofactors like UV light and chemical carcinogens will be primary candidates to be tested in the newly developed system. All this promises new insights into the papillomavirus host interactions in dogs but also in general and can help to deal with the various types of papillomas more sophisticated in the future. The technique may furthermore be applied to generate similar cell lines for other papillomaviruses in species of interest.

Our identification of novel papillomaviruses in distinct papillomatoses in horses goes in line with the hypotheses we were able to support in dogs and that had previously been suggested and supported in cattle (Campo, 1984; Hatama, 2008; Hatama 2009). We were able to link the known but etiologically poorly characterized penile papillomas and the aural plaques to papillomaviruses (Scott, 2003) (pages 67-73). These very distinct disorders seem to be associated with different papillomavirus types, and there is evidence that EcPV2 comes in at least two different variants. Also the three now known equine papillomaviruses represent three different phylogenetic genera. As in dogs the sequencing of further papillomavirus types

and also subtypes and variants is desirable to gain more understanding about the diseases and about papillomavirus evolution. Especially the surprising finding that there may be a kind of monophylogeny in equine papillomaviruses encourages the search for further papillomaviruses of horses as well as of other equide species to improve the resolution of the phylogenetic tree.

In horses as well as in dogs the DNA of certain papillomaviruses can be identified on the clinically unaffected skin (Vanderstraeten, 2011). We therefore intended to locate the viral DNA in the relatively common EcPV2 associated lesions of the penis (pages 74-85). The correlation of the presence of viral DNA with typically virus associated cell alterations supports causal relation between lesion and virus and is further supported by independent results of similar studies (Scase, 2010). However looking for a correlation between viral particles, DNA and RNA in such lesions would be a desirable approach to assess causality in the future. Further experiments should also address questions about the distribution of the virus among the horse population using for example PCR and ELISA techniques in combination and also the role of the genetic variability of EcPV2 would be worth investigating in more detail.

Taken together our studies on canine and equine papillomaviruses and papillomatoses have indicated several associations between viruses and disorders, supported previous hypotheses and established new methods. They therefore can be seen as unique parts of the papillomavirus puzzle. Many new questions have arisen and further studies are warranted for which the described ones have paved a way.

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